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**WO 01/57530**

(54) Title: METHOD AND APPARATUS FOR SIGNAL TRANSDUCTION PATHWAY PROFILING

(57) Abstract: An assay device for determining the presence of analytes in a cell lysate comprises a porous support member and a plurality of binding reagents arranged and immobilized at multiple reaction sites on the support member. The binding reagents are selected and arranged to assess the status of a selected cellular signal transduction pathway/protein-protein interactive network. In a further aspect, a method for assessing the status of a signal transduction pathway comprises generating a lysate of cells, the lysate retaining one or more pathway molecules present in one or more states and the pathway molecules reflecting signal transduction events taking place in the cells. The method further includes applying the lysate to an immobilized series of binding reagents which can discriminate the pathway molecules and their states. Binding events between the pathway molecules and the binding reagents are identified and the state of the selected signal pathway is determined.

METHOD AND APPARATUS FOR SIGNAL  
TRANSDUCTION PATHWAY PROFILING

RELATED APPLICATION

5        This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application Serial No. 60/179,997, filed February 3, 2000. U.S. provisional application Serial No. 60/179,997 is incorporated herein by reference in its entirety.

10        BACKGROUND OF THE INVENTION

The present invention relates to an apparatus and method for assessing the status of a cellular pathway. Several biotechnology companies are developing DNA-chips which contain printed arrays of hundreds or thousands of 15 genes, or more. However, the DNA sequence of the genes or the level of gene expression do not reflect the actual functional state of the proteins encoded by the genes. The functional state of each protein involves more than simply the amount of the proteins. It includes, for example, post 20 translational modifications (phosphorylation, glycosylation, etc.), binding partners in cellular pathways, conformation, enzymatic function, and state of activation. Moreover, each cell in the body has a pattern of functional proteins which reflects the current biologic working state of the cell 25 (e.g., growing, differentiating, diseased, dying, senescent, etc.). Therefore, there exists a need in the art for a device and method for determining the status of cellular pathways (e.g., the phosphorylation state or binding partners of involved proteins) in tissue samples, including human 30 tissue samples, thus providing the diagnostician or clinician with an early warning of impending or occult toxicity,

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disease state, response to treatment, differentiated function, and so forth.

#### SUMMARY OF THE INVENTION

The present invention provides a molecular detection device having a plurality of binding or reaction sites comprising a series of immobilized recognition molecules or binding reagents selected and arranged to qualitatively or quantitatively assess the status of a series of signal transduction pathway proteins or other protein-protein interactive network, their phosphorylated or activated state, and their binding partners, in a cell sample to determine the status of a selected cellular signal transduction pathway. Knowledge of the pathway status can then be used by the diagnostician or clinician to determine the health of the sampled cells, drug or other treatment efficacy or toxicity. This knowledge can also be used to identify candidates for selected therapies, to aid in therapy selection for a given subject, and to determine drug toxicities.

Protein interactions are defined herein as the coupling of two or more proteins in a given space and time such that a binding reaction occurs, and/or one protein modifies the other protein. The novel concept of the invention is that the protein networks and pathways, and their changing state in cells, can be recapitulated after the cells are dissolved and the proteins are solubilized. The invention method recapitulates the proteins involved in networks because a) networks are built from proteins binding to other proteins to form larger complexes; and b) phosphorylation events cause specific proteins to interact with (or dissociate from) other proteins in a certain order. Therefore, the upstream and downstream networks emanating from any given point can be elucidated by using the subject invention to find at least two proteins that are phosphorylated and forming complexes with other proteins. This pattern changes with disease state or drug treatment or toxicity.

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In a preferred embodiment, multiple nodes in a signal transduction pathway circuit within a cell are profiled to determine whether the entire pathway is functionally activated and in use by the cell or whether only 5 a portion of the pathway is activated (e.g., upstream or downstream), thus indicating, for example, that the pathway may be regulated at intermediate points along the pathway circuit. Also, the binding partners which complex with individual nodes of the pathway can be identified.

10 In still a further aspect, a method of identifying the full repertoire of proteins that could serve as acceptors for phosphorylation comprises treating cells, such as whole tissue specimens grown ex-vivo, or animal tissue treated in vivo with compounds that inhibit protein tyrosine and/or 15 serine/threonine phosphatase activity such as sodium pervanadate, okadaic acid, calyculin A, for acute periods of time (e.g., less than 3 hours), and isolating the cells of interest. The cells of interest are lysed and selected for the phosphorylated proteins using antibodies on an 20 immobilized bait, such as anti-phosphotyrosine, anti-phosphoserine, and/or anti-phosphothreonine antibodies, and so forth. The phosphoproteins in the enriched fraction are separated, e.g., by chromatographic and/or electrophoretic means and the primary amino acid sequence of the proteins is 25 identified by enzymatic digestion or chemically-induced protein fragmentation followed by standard mass spectrometric techniques. Finally, antibodies or binding molecules that specifically bind to the identified proteins are developed.

In further embodiments, protein pathways which 30 reflect a disease state can be fingerprinted to pinpoint a therapeutic target. In order to select a treatment or determine treatment efficacy, informatics or heuristics can be developed for various pathways, pathological states, therapies, and the like.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

The invention may take form in various components and arrangements of components, and in various steps and arrangements of steps. The drawings are only for purposes of illustrating preferred embodiments and are not to be construed as limiting the invention.

FIG. 1 illustrates a cellular signal transduction pathway profiling device of the present invention.

FIGS. 2-6 illustrate exemplary pathway profiling methods in accordance with the present invention.

FIG. 7 illustrates a system and method for identifying therapeutic targets employing trapped lysates from cells of different pathological states.

FIG. 8 shows a multiplexed embodiment of the molecular detection device in accordance with the present invention.

FIG. 9 illustrates an exemplary pathway profiling device according to the present invention.

FIG. 10 illustrates autoradiography results for microdissected erb2+ and erb2-human breast cancer epithelium from tissue sample specimens probed for anti-ERK1/2 mouse monoclonal antibody using the pathway profiling device according to FIG. 9.

FIG. 11 illustrates a method of the present invention for the detection of IFNA stimulation of the JAK-STAT signal pathway.

FIG. 12 illustrates the use of nitrocellulose engraved by a laser to manufacture raised porous columns that contain and channel the flow of cellular proteins through the zones. In the depicted example, a high sensitivity and dose dependence of protein detection is noted for the intracellular human heart muscle protein troponin.

FIGS. 13A-13C, 14A, 14B, and 15-18 illustrate the use of activated glass beads as the flow-through matrix of the present invention.

FIG. 19 is a flow chart of a signal transduction pathway profiling method in accordance with the present invention.

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FIGS. 20 and 21 illustrate the use of patterns for the identification of a pathway and/or disease state of a cell sample.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 Referring now to FIG. 1, a cellular signal transduction pathway profiling device 100 includes a porous support member 110, such as a porous membrane or the like. Support member 110 may be fabricated from any material into which fluids may flow and readily pass through, such as  
10 nitrocellulose, cellulose, glass, nylon, or other fibrous material. On the support 110, two or more, preferably three or more, distinct binding site regions or trapping zones 116 are formed by applying and immobilizing within each region a substance capable of reaction with an analyte contained  
15 within the test sample. In a preferred embodiment, each region contains a single purified ligand, such as a protein, peptide, antibody, or drug capable of trapping a specific protein or modification thereof involved in a selected transduction pathway. Alternately, the immobilized materials  
20 at the binding sites are complex mixtures (e.g., cellular lysates). By "immobilized" is meant that the substance capable of holding an analyte is applied in a confined area on the surface of the membrane such that it is permanently bound or otherwise incapable of substantial movement to  
25 positions elsewhere on the support.

A lysate application region 114 and a chase application region 112 are provided at one end of the device 100. In this manner the lysate can be applied to the support 110, followed by a chase, such as a buffer or other  
30 physiological solution, to cause the lysate to flow through the trapping zones 116 to an outflow region 118. Preferably, the lysate is drawn through the reaction zones via wicking or capillary action, although other methods are contemplated as well, such as gravity, application of a pressure  
35 differential, electrical pumping, and so forth. The trapping zones are applied in a manner such that subsequent capillary wicking of material for analysis will pass through the

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immobilized protein zone and not around it. In one embodiment, the support material 110 forms a strip and the reaction zones 116 are applied in a line traversing the width of the strip.

5 When antibodies are used for trapping, they may be from any species, including but not limited to human, goat, rabbit, mouse, etc. The antibodies can be either anti-protein specific (e.g., anti-ERK kinase) or anti-modified protein specific (e.g., anti-phosphorylated ERK kinase).

10 For signal transduction profiling, the sequence of the applied antibodies in the multiple reaction zones 116 are preferably ordered such that the protein components of a pathway that are activated last are captured first, although the ordering is otherwise not necessarily critical. For 15 example, the analysis of EGF signaling profiles from cells that are expressing differential amount of the erb receptor family can be analyzed by imprinting antibodies recognizing the phosphorylated forms of proteins in the EGF receptor signaling cascade. The order of imprinting, from first 20 capture zone to last capture zone, is shown below in TABLE 1.

TABLE 1

	Capture Zone	Antibody
	1	anti-phosphorylated c-myc
	2	anti-phosphorylated c-jun
25	3	anti-phosphorylated p70RSK
	4	anti-phosphorylated erk
	5	anti-phosphorylated AKT
	6	anti-phosphorylated c-RAF
	7	anti-phosphorylated erb2
30	8	anti-phosphorylated erb1

Once the trapping zones 116 have been imprinted on the support 110, cellular lysates, DNA aptomer libraries, focussed or unfocussed drug libraries, and/or phage display libraries, can be applied to the top of the detection device

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100 in the defined application zone 114 and allowed to actively wick through the support 110 by active capillary action using a buffer chase applied to region 112. Again, other methods of drawing the lysate through the zones 116 are 5 also contemplated.

Analytes in the material for analysis, such as proteins, drugs, DNA, phages, will, depending on their abundance, specifically bind to a corresponding trapping zone region 116 having a substance capable of reaction therewith. 10 All other components will wick through the zone into the waste outlet region 118 at the bottom of the chip 100.

Because these analyses are performed under native conditions, protein complexes, comprising activated proteins (e.g., phosphorylated proteins) and their binding partners 15 will be trapped in each of the subsequent zones, depending on the degree of phosphorylation and the presence of the binding partners.

Subsequent analysis and/or identification of proteins trapped in the zones 116 is performed by 20 countercurrent extraction, treatment with enzymes (e.g., trypsin), mimetics (e.g., phenylphosphate for the removal of phosphotyrosine-containing proteins), and so forth. Analysis of extracted material can be performed by elution into other trap zones (three-dimensional separation) or by mass 25 spectrometry (e.g., LCQ-MS, CE-ESI-MS) for identification and discovery purposes. Additionally, quantitative analysis of trapped analyte composition can be performed by querying each trap zone with a tagged or detectably labeled antibody (e.g., biotinylated or alkaline phosphatase tagged) to generate a 30 signal. Exemplary methods for detecting the bound proteins are illustrated in FIGS. 2-6.

An exemplary embodiment in which each trapping zone 116 is imprinted with lysates from cells of different types or different pathological states is illustrated in FIG. 7. 35 Phage and/or DNA aptamer libraries 220, such as random phage and/or random aptamer libraries, can be screened and analyzed by successive rounds of trapping with a plurality of protein lysate zones 216a-216c. For example, in a first binding site

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**216a**, there is applied and immobilized a normal cell lysate. In a second binding site **216b**, there is applied and immobilized a lysate of cells in a predisease state. In a third binding site **216c**, there is applied and immobilized a lysate of diseased cells. Each round of trapping is followed by amplification. For example, each binding site can be cut out and amplified using PCR (for aptomer libraries) or amplification by infection (e.g., in *E. coli* for phage display peptide libraries). Alternately, small molecule drugs can be identified by NMR or other like methodologies. The amplified entities **222** can then be used for potential targeting therapies, e.g., as toxin-conjugated vehicles. For example, an amplified entity which selectively binds to a protein representative of a disease state, such as cancer, can be conjugated or linked to a toxin for efficient delivery of the vehicle to the targeted cells. Likewise, an amplified entity can be conjugated to an imaging reagent for diagnostic imaging of the targeted cells, such as x-ray contrast agents, MRI contrast agents, radiopharmaceuticals for nuclear medicine diagnostic imaging, and so forth. These entities can be further screened for DNA or peptide sequences that bind to unique cell-specific or tissue-specific proteins by repanning against immobilized bait trap surfaces.

Referring now to FIG. 8, there is shown another embodiment of the present invention in which a molecular detection device **300** comprises a plurality of support members **310** arranged in a multiplexed format. Each support member includes a plurality of binding sites **316** to which is applied and immobilized a series of proteins and modifications thereof involved in a selected signal transduction pathway. Such a format is suitable for high-throughput drug screening using cell lines and/or microdissected tissue cell lysates. A lysate application zone **314** is provided for each strip **310**. In alternate embodiments, the zones **314** are cell growth zones for selected cell lines, in which case an optional lysing buffer application zone **322** is provided such that the cells are lysed prior to reaching the trapping zones **316**. A chase application zone **312** is provided for application of a buffer

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or other physiological solution to carry the lysate through the trapping regions 316. Analytes in the lysate will specifically bind to a corresponding trapping zone region 316 having a substance capable of reaction therewith and all 5 other components will wick or otherwise be drawn through the zones into the waste outlet region 118 at the bottom of the device 300.

The reduction of this method to practice has been demonstrated in the following tests. A pure nitrocellulose 10 membrane (0.45 micron pore size, Shleister and Schuell) was cut as a 6 cm x 1 cm strip beginning and ending with wider 3 cm x 3 cm ends. The following commercially available rabbit polyclonal antibodies (New England Biolabs, Upstate Biotechnology) at 100 microgram/ml concentration were applied 15 undiluted in total applied volume of 2 microliters as trapping zone "stripes" perpendicular to the length of the membrane as shown in FIG. 9. The antibodies were applied in bands approximately 2-3 mm wide, spaced at repeated 0.5 cm intervals, and ordered from top to bottom as shown in TABLE 20 2.

TABLE 2

	Capture Zone	Antibody
25	1	anti-phosphorylated c-myc
	2	anti-phosphorylated p70 SK kinase
	3	anti-phosphorylated elk
	4	anti-phosphorylated STAT5
	5	anti-phosphorylated AKT
	6	anti-phosphorylated c-erb2
	7	anti-phosphorylated c-erb1
	8	anti-phosphorylated erk kinase

The antibodies were allowed to bind to the membrane overnight, after which the entire strip was immersed in a commercially available casein blocking solution (Superblock, Pierce Chemical) for 2 hours. The membrane was then washed 35 3 times for 10 minutes with 20 milliliters of a 50 mM TRIS,

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100 mM NaCl, 0.5% Tween-20 solution (TBST). The strip was then allowed to air-dry for 5 hours.

Lysates comprised of 1500 cells procured via Laser Capture Microdissection (LCM) of two human breast cancer specimens, one known to be highly reactive for erb2 (erb2+), and the other known to be weakly reactive (erb2-), were applied to the top of the strip (lysate application region) in a volume of 5 microliters of commercially available lysing buffer (T-PER, Pierce Chemical) with a commercially available protease inhibitor cocktail (Complete Tablets, Boehringer Mannheim) and 1  $\mu$ l sodium vanadate as a phosphotyrosine phosphatase inhibitor. 25 microliters of the T-PER solution was applied as a chase immediately after the application of the lysate, and the lysates were allowed to actively "wick" through the strip over a period of 60 minutes. An additional volume of 25 microliters of T-PER was applied to the chase zone when the buffer front was approximately 1/2 of the way through the strip.

Five minutes after the buffer front had passed through the last antibody trap zone, the entire strip was immersed and washed 3 times for 10 minutes in TBST. The strip was then immersed in TBST + mouse monoclonal anti-ERK (Transduction Laboratories) at a 1:2000 dilution for 1 hour. The strip was then washed 3 times for 10 minutes each time with TBST, and then incubated for 1 hour with a biotinylated goat anti-mouse IgG antibody (Vector Laboratories) at a 1:5000 dilution.

The strip was then washed 3 times for 10 minutes each time in TBST, and developed according to the package insert from Vector Laboratories using a commercially available kit (ABC reagent) which generates luminescent output. The strips were then exposed to standard autoradiography for 1 second to 30 second exposures. The results of the autoradiography are illustrated in FIG. 10.

The following experimental example demonstrates the recapitulation of the state of a cellular signal pathway using proteins dissociated from lysed human cells treated with a drug known to activate the selected signal pathway.

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The Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway is important in the Interferon Alpha (IFNA) cellular response. Binding of IFNA induces the following sequence of events:

- 5      Fusion of Interferon Alpha Receptor(IFNAR)1 and IFNAR2; Jak1 and Tyk2 are phosphorylated;
- IFNAR1 is phosphorylated allowing docking of Stat2;
- Stat2 is phosphorylated allowing Stat1 to dock;
- Stat1 is phosphorylated;
- 10     Stat1(Pi)-Stat2(Pi) heterodimer is released.

#### Interferon Alpha Treatment of Peripheral Blood Lymphocytes

Peripheral Blood Lymphocytes (PBLs) were isolated using Histopaque-1077 (Sigma). The PBLs were suspended in PBS (0.01 M phosphate buffered saline, 0.138 M NaCl, 0.0027 M KCl), pH 7.4, and allowed to acquiesce overnight at 4°C. The cells were pelleted by centrifugation at 5000xg for 5 minutes. The supernatant was decanted off, and the cells resuspended in RPMI-1640 media (Bio-Whittaker) at approximately 20 million cells/mL. The cells were treated with 13,500 units/mL of recombinant human interferon alpha (BioSource) for 0, 3, 10 or 30 minutes. Once the incubation was complete, cells were pelleted by centrifugation at 5000xg for 5 minutes. Cells were washed with ice-cold PBS and resuspended in lysis buffer ((T-PER, Pierce), 10 mM beta-glycerophosphate, 1 mM sodium molybdate, 2 mM sodium orthovanadate, 2.5 mM AEBSF and 5% glycerol). The cells were vortexed and placed on ice for 5 minutes, repeated. Lysates were clarified by centrifugation at 10,000xg for 5 minutes.

30    The supernatant was snap frozen and stored at -80°C.

#### Pervanadate Treatment of Peripheral Blood Lymphocytes

Peripheral Blood Lymphocytes (PBLs) were isolated using Histopaque-1077. The PBLs were suspended in PBS and allowed to acquiesce overnight at 4°C. The cells were pelleted by centrifugation at 5000xg for 5 minutes. The

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supernatant was decanted off, and the cells resuspended in RPMI-1640 media at approximately 20 million cells/mL. A pervanadate solution was made as follows. 882  $\mu$ L of 0.10 M sodium orthovanadate was added to a mixture containing 832  $\mu$ L 5 RPMI-1640 media and 50  $\mu$ L 30% H<sub>2</sub>O<sub>2</sub>, mixed at room temperature, and let stand for 15 minutes. The cells were treated with pervanadate 1  $\mu$ L to 500  $\mu$ L of cells for 30 minutes at 37°C. Cells were pelleted at 5700xg for 5 minutes. Pellets were washed with ice-cold PBS and snap frozen. T-PER (salt 10 concentration was adjusted from 0.150 M to 0.20 M) was added to the pellet and the resulting lysate was vortexed rapidly for 1 minute. The lysate was clarified by centrifugation at 15,000xg for 10 minutes. The supernatant was snap frozen and stored at -80°C.

15                 Preparation of Flow-Through Trapping Zones.

FF85 (Schleicher and Schuell) nitrocellulose membranes were cut into approximately 8 cm x 1 cm strips. Stat2 antibody (C-20, Santa Cruz Biotechnology) was spotted down at 0.5  $\mu$ L increments/2.5  $\mu$ L total across the 1 cm width 20 of the strip at approximately 6.0 cm from the top. Stat1 $\alpha$ p91 antibody (C-24, Santa Cruz Biotechnology) was striped down at 0.5  $\mu$ L increments/2.5  $\mu$ L total across the 1cm width of the strip at approximately 7.0 cm from the top. The treated membranes were dried for 45 minutes at room temperature and 25 6% relative humidity. The strips were blocked in Superblock (Pierce Chemical Company) for 3 hours at room temperature with shaking. The strips were washed 3 times with TBS-T (0.20 M Tris, 0.50 M NaCl, 0.1% Tween20), pH7.5, then dried at 37°C/6% relative humidity.

30                 p-Stat1 Association Strip Assay

The IFNA treated PBL lysates (25  $\mu$ L) were placed approximately 1 cm from the top of the strip. The lysate was moved through the strip upon addition of TBS-T to the top of the strip. Every 20 minutes over a period of 3 hours, 50  $\mu$ L 35 of TBS-T was placed at the top of the strip. Upon completion the entire strip was washed 1 time with TBS-T. The strips

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were incubated overnight at 4°C with p-Stat1 (A-2, Santa Cruz Biotechnology) diluted at an appropriate concentration in Superblock. The strips were washed 3 times with TBS-T. The strips were then incubated at room temperature for 3 hours  
5 with Goat Anti-Mouse:Alkaline Phosphatase (Fortran, In House Conjugation to AP) diluted in Superblock. The strips were washed 3 times with TBS-T. The strips were incubated with CDP-Star (Tropix) for 5 minutes. Light output/binding of p-Stat1 antibody was recorded using a CCD imager (NightOwl,  
10 Berthold Technologies). As shown in FIG. 11, the invention method was able to detect IFNA stimulation of the JAK-STAT signal pathway in the expected time-dependent manner.

#### CTNI Example

The following Example demonstrates how parallel  
15 arrays of flow-through binding zones useful for practicing the subject invention can be manufactured by using a laser engraver to build up porous matrix columns. The raised columns on a plastic backing contain and direct the flow of solubilized proteins to be analyzed. The example indicates  
20 the quantitative dose-dependent detection of troponin, an intracellular myofibril component of human cardiac muscle cells.

Nitrocellulose, purchased from Schleicher and Schuell, comprises an inert plastic (Mylar) backing support  
25 onto which a layer of directly cast nitrocellulose is deposited. During our investigation of this matrix for flow-through diagnostic applications, it was discovered that it is possible to create channels in the material. This was achieved by using the cutting power of a CO<sub>2</sub> laser. The laser  
30 is programmable through a computer software application, which enables the creation of highly complex patterns on the surface of the nitrocellulose. Briefly, the laser vaporizes the cellulose beneath the beam, exposing the underlying mylar surface. One of the simplest applications of this process is  
35 to take a 4 cm x 4 cm square of nitrocellulose (or any other desired size) and use the laser to cut a series of vertical slots in the material. In this manner, one creates in the

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- nitrocellulose a number of "columns", each column separated by an inert mylar barrier. Interpreted another way, a series of "usable" ridges are manufactured on the matrix, each ridge usable for a similar or dissimilar diagnostic application.
- 5 The number of ridges created on the material varies with the width of the ridge and the length of the nitrocellulose piece. The laser action also scores the upper surface of the mylar, allowing for easy detachment of a single column or multiple columns from the sheet.
- 10 A series of columns (3 mm wide) were cut into a 4 cm x 4 cm length of nitrocellulose. Seven columns were detached and treated as follows.
- 15 1  $\mu$ l of a 1 mg/ml solution of neutravidin (Pierce Chemical Co., Rockford, Illinois) was applied to each ridge.
- 20 The protein was heat (37\*) cured on the matrix for 2 hrs. After washing, a 1  $\mu$ l aliquot of biotinylated anti-cardiac troponin I antibody was overlayed onto the original neutravidin spot. Thirty minutes later, excess antibody was removed and the whole surface of the matrix blocked in a PEG/PVP solution.
- A series of troponin I calibrators (purified from human heart muscle), ranging in concentration from 0 to 160 ng/ml were reacted individually with an anti-troponin I antibody conjugate. The latter recognizes a site on troponin I, which differs from the site recognized by the neutravidin bound antibody.
- 25 A 1  $\mu$ l aliquot of each antigen/conjugate reaction was placed onto individual columns. The placement of the liquid was immediately below the neutravidin complex.
- 30 The lower edge of the seven column composite was placed into a buffer allowing the latter to move through the material via capillary action (conventional TLC). The length of time required for the buffer to reach the top of the device was 45 seconds.
- 35 The device was removed and coated in CDP\* substrate (Tropix Corporation, Bedford, Massachusetts), light emission was recorded on a Nightowl CCD scanner of light emission.

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The flow-through trapping zones created in nitrocellulose porous matrix columns efficiently retained antibody antigen complexes such that the troponin analyte could be measured in a dose-dependent manner as shown in FIG.

5 12 and TABLE 3.

TABLE 3

<u>Card Style Troponin Membrane; Dose Curve 1 <math>\mu</math>L(Sample + Conjugate)</u>		
	<u>Troponin (ng/mL)</u>	<u>Light output (cts/sec)</u>
	0	0
10	2.6	4955
	5.2	7264
	10.4	8477
	20.8	13116
	41.65	18199
15	83.5	21415

The porous matrix material through which the cellular protein flows has been successfully reduced to practice with porous or fibrous materials, such as nylon, cellulose or silica, and can be configured as raised porous 20 interconnecting columns or suitably packed particles or beads.

The following example demonstrated the use of activated glass beads to construct a flow through matrix of the present invention.

25 Controlled pore glass (CPG) is a matrix whose surface is readily modified by reaction with a wide variety of bifunctional silanes. The material exhibits a significant surface area to weight ratio. The rigid nature of the glass, high porosity and non-compressibility lends itself to rapid 30 passage of liquids or biological fluids through the matrix. The present invention employing a series of capture zones having differing specificity for removal and quantitation of phosphorylated and non-phosphorylated protein complexes from stimulated cells can be achieved through the use of CPG.

35 By confining specific capture zones of CPG in a micro-capillary tube, each zone separated by either inert

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glass or an inert cellulose plug, biological fluids can be drawn through each zone (e.g., using a vacuum or positive displacement pump, or the like), to capture the desired entities in the zones. The microenvironment of the capillary  
5 minimizes diffusion limitations thereby enhancing acceleration of the rate of binding of the ligand to the capturing agent.

Accordingly, CPG (Sigma Corporation, St. Louis, Missouri) was reacted with 3-aminopropyl trimethoxysilane  
10 (Sigma Corporation, St. Louis, Missouri) using standard published procedures. Subsequently, the amino modified glass was reacted with iminothiolane (Traut's reagent, Pierce Chemical Co., Rockford, Illinois). The latter procedure provides a sulphhydryl group (thiol) for further reaction with  
15 maleimido modified proteins, drugs, nucleotides and other entities so modified.

Maleimido horseradish peroxidase was reacted with 20 mg of iminothiolane CPG. After washing, small portions (2 mg x 3) of the beads were loaded into a micro-capillary tube.  
20 Each zone was separated by an inert glass or plug. A chemiluminescent substrate (Duolux, Lumigen, Southfield, Michigan) was pulled rapidly into the capillary using vacuum. The tube was transferred to a light measuring device (NightOwl CCD light scanner) and the light output read for 1  
25 sec. The results are shown in FIG. 13A. In either case, three illumination zones are indicated, thereby demonstrating the feasibility of the system. In a further iteration, an epitope of cardiac troponin I (P14 cTNI epitope, Research Genetics, Huntsville, Alabama), conjugated to thyroglobulin  
30 (porcine thyroglobulin, Sigma Corporation, St. Louis, Missouri) was reacted with CPG. An antibody (P3 cTNI antibody, Fortron Bioscience Inc., Morrisville, North Carolina), conjugated to peroxidase or alkaline phosphatase (AP), was introduced into the requisite capillary, excess  
35 conjugate was removed through rapid washing and the bound antibody enzyme visualized with an appropriate chemiluminescent substrate. The results are recorded in FIGS. 13B and 13C.

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A single peroxidase bead, 100-200 microns in diameter, was exposed to Duolux and the light output recorded, compared with substrate background. The calculated signal to noise ratio was 3.5, indicating that the system has  
5 the potential of being reasonably sensitive. The results are shown in FIG. 14A.

In order to provide a generic CPG system, maleimido-neutravidin (neutravidin and maleimido peroxidase were purchased from Pierce Chemical Co., Rockford, Illinois)  
10 was reacted with iminothiolane glass. Neutravidin binds biotinylated species aggressively. Such biotinylated species include peptides, proteins, drugs, nucleotides and other entities so modified. In this example, biotin containing Stat 1 and phosphorylated Stat 1 antibodies (Stat1{pY<sup>701</sup>}  
15 peptide, BioSource International, Camarillo, California) were attached to CPG. Bovine serum albumin (Sigma Corporation, St. Louis, Missouri) containing phosphorylated Stat 1 peptide was drawn into a capillary, in which a zone of each was antibody was deposited. After washing, the zones were probed  
20 with RC20-AP (RC20-AP antibody conjugate purchased from Transduction Laboratories, Lexington, Kentucky). Following a further wash, substrate was introduced and the light output recorded. In this experiment it was anticipated that the luminescence from the p-Stat 1 zone would be greater than  
25 that from the Stat 1 zone. This turned out to be the case. The light output from the Stat1 zone is related to the poor quality of the RC-20 AP conjugate and probably represents non-specific binding to the bound matrix antibody. To demonstrate that binding to a zone is reversible in the  
30 presence of an appropriate ligand, p-Stat 1 antibody beads were exposed to p-Stat 1 peptide peroxidase, both in the absence and presence of p-Stat1 peptide albumin (blocking agent). As anticipated, the presence of the peptide reduced the binding of the conjugate, resulting in less light output.  
35 The results are shown in FIG. 14B.

To further demonstrate the utility of our CPG capillary approach, neutravidin beads were reacted with a biotinylated antibody which recognizes a specific epitope on

- 18 -

cardiac troponin I (cTNI). A negative serum sample was drawn through the capillary (vacuum). After washing, a secondary AP labeled antibody, with a different specificity for cTNI was introduced and excess conjugate removed immediately by 5 washing. Exposure of the beads to substrate produced the light output shown in FIG. 15 (-♦-). Using the same capillary and procedure, a positive sample of cTNI (490 pg/ml) was examined for light yield (-■-). The calculated signal to noise ratio of 5 to 1 indicates that the system is 10 capable of extreme sensitivity. An overlay of the light output from the capillary is included in FIG. 15.

Peripheral blood lymphocytes (PBLs) were exposed to alpha-interferon. The cells were lysed and cellular debris removed by centrifugation. In a similar fashion, non-treated 15 cells were and lysed and the cell contents collected. Fifty microliters of each lysate were drawn through separate capillaries, each containing Stat 1 antibody CPG beads. The capillaries were probed with RC20-AP. After washing, substrate (CDP \*) was drawn into the tubes and the light 20 emission recorded in a tube luminometer (Junior Luminometer, Berthold Technologies) or the NightOwl CCD scanner. The data indicates that stimulation of PBLs with interferon, induces an increase of phosphorylated tyrosine species, which bind to Stat 1 protein. This is indicative of binding of tyrosine 25 phosphorylated Stat 2 to Stat 1, thus forming the recognized heterodimer, known to be part of the alpha-interferon/lymphocyte signaling cascade. The results are illustrated in FIG. 16

An additional approach to confirm the observations 30 from FIG. 16 is described here and shown in FIG. 17. Instead of probing with RC20-AP, a non-labeled mouse antibody specific for phosphorylated tyrosine in conjunction with a goat anti-mouse AP conjugate were employed sequentially. Negatively and positively, alpha-interferon stimulated PBL 35 lysates were drawn through individually packed capillaries containing Stat 1 antibody CPG. After washing, etc., and exposure to the primary and secondary antibodies, the beads were exposed to CDP\* substrate. The increase in positive

- 19 -

light emission supports our contention that stimulation of PBLs with alpha-interferon leads to an increase in phosphorylated Stat2, which in turn binds to Stat 1 protein.

The phosphatase activity of PBLs is significantly reduced in the presence of pervanadate. Inhibition of phosphatase activity enhances the overall level of phosphorylated proteins within the lymphocyte. The experiment shown and described with reference FIG. 18 illustrates this phenomenon quite nicely. Briefly, CPG Stat 1 antibody beads (10 mg), were placed in a Z-spin well (Z-spin columns were obtained from Fisher Scientific, Pittsburgh, Pennsylvania). As a control, plain neutravidin CPG was used. To each well, 50 µl of pervanadate treated PBL lysate was added. The wells were centrifuged, washed and then exposed sequentially to mouse anti-phosphorylated tyrosine antibody and goat anti-mouse AP conjugate. Each addition was followed by a wash/centrifuge cycle. The addition of CDP\* substrate induced light emission which was recorded in the NightOwl. The data indicates that inhibition of phosphatase activity enables measurement of the basal level of phosphotyrosine proteins in non stimulated PBLs. In this instance, the data suggest that this type of methodology is capable of discriminating phosphorylated Stat2 from a whole range of known and as yet unknown phosphorylated signaling intermediates.

The following are experimental examples of more than 20 specific different trapping ligands that were successfully used in the subject invention for protein network profiling. The invention methods led to identification of phosphorylated proteins and assignment of their functional participation in signal pathways. The information derived is relevant to the following: a) identifying specific protein-protein interactions; b) assignment of the specific interactions into larger functional circuits and networks; c) downstream ordering of protein components in a given pathway; d) specific disruptions caused by drug treatments, disease, or toxicity; and e) identifying patterns of protein interactions or

- 20 -

phosphorylations that are unique to disease state, drug treatment or toxicity.

1. 100,000 primary human lymphocytes and 100,000 primary human monocytes obtained by tangential centrifugal 5 elutriation and differential ficoll-percoll gradient selection were resuspended in 5 ml (each cell type) RPMI-1640 media.

2. 1 ml aliquots were placed in eppendorf tubes

3. A 100X cocktail (herein referred to as PI or 10 phosphatase inhibitor cocktail) consisting of the following: 2500 nM Okadaic acid (cell-permeable inhibitor of protein phosphatase); 500 micromolar sodium pervandate (membrane soluble form of sodium orthovanadate and an inhibitor of tyrosine phosphatases), 500 nM Calyculin A (cell-permeable 15 inhibitor of protein phosphatase 2A and protein phosphatase); and 2500 micromolar phenylarsine oxide (cell-permeable phosphotyrosine phosphatase inhibitor).

4. The following experimental treatment protocol was then set up and performed with each 1 ml cell suspension 20 (20,000 cells/ml):

i. primary human lymphocytes/monocytes + vehicle alone (10 microliters DMSO) 1 hour;

ii. primary human lymphocytes/monocytes + 1X PI 1/2 hour; and

25 iii. primary human lymphocytes/monocytes + 1X PI 1/2 hour + 10 micromolar SB 203589 (p38 kinase inhibitor) 1/2 hour.

5. Cells were pulse centrifuged at 5000xg for 2 minutes, washed twice in ice cold PBS and the resultant cell 30 pellet lysed in 100 microliters lysing solution (TPER (Pierce Chemical) + 2 mM sodium orthovanadate (tyrosine phosphatase inhibitor), 10 mM B-glycerol phosphate (serine phosphatase inhibitor), 300 mM NaCl, 4 NM AEBSF (protease inhibitor)), vortexed rapidly for 1 minute and clarified by centrifugation 35 for 5 minutes.

At this point the lysates are analyzed by 4 main methods.

- 21 -

i. Open faced array of antibodies immobilized on nitrocellulose (see No. 6 list below).

ii. Flow-through devices striped with trapping zones containing protein binding ligands.

5 The analysis was complemented by the following additional standard methods:

iii. Two-dimensional or one-dimensional gel-based separation technologies.

iv. Mass-spectrophotometric-based baiting tech-  
10 nologies.

6. The resultant whole cell lysate was then incubated overnight at 4 degrees C on an Oncyte multiwell (Grace Biolabs, Inc) nitrocellulose slide that was spotted with rabbit polyclonal phospho-specific antibodies  
15 recognizing the following 20 proteins: EGFR (Tyr1173); Tyk2 (Tyr1054/1055); ATF-2 (Thr71); eIF2a (Ser51); eIF4E (Ser209); Cdc2 (Tyr15); p38 (Thr180/Tyr182); Cdk1 (Thr14/Tyr15); CREB (Ser133); Akt (Ser473); eNOS (Ser1177); CREB (Ser133); c-Jun(Ser63); Elk-1 (Ser383); ErbB2 (Tyr1248); Bad (Ser112);  
20 Jak1 (Tyr1022/1023); p70 S6 (Thr421/Ser424); ERK1/2 (Thr202/Tyr204); and Cdc25 (Ser216).

7. The slide was washed 3x with TBC-tween for 5 minutes, then incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody for 1 hour at 4 degrees  
25 C.

8. Chemiluminescent detection was performed using the ECL-plus kit from Amersham following recommended manufacturer procedures.

9. Proteins whose phosphorylation is dependent  
30 upon p38 kinase activity and lie downstream of the p38 kinase are subsequently identified by their inhibition of phosphorylation due to the pre-incubation of the specific p38 kinase inhibitor.

10. For 2D-PAGE applications, the lysates are  
35 enriched for phosphorylated proteins via:

i. Affinity chromatography-based or immuno-precipitation-based methods (i.e., using a anti-

- 22 -

phosphotyrosine antibody, anti-phosphoserine antibody, etc.); and

5 ii. Eluted by a competitive mimetic such as phenylphosphate or sodium pyrophosphate or a specific peptide used as the antigen for the development of the antibody.

10 iii. The resultant eluate is then run on the gels directly, and phosphorylated proteins detected by traditional staining procedures compatible with mass spectroscopy-based protein identification (e.g., colloidal coomassie,ponceau S, SYPRO Ruby-red (Molecular Probes, Eugene, OR), and so forth).

15 iv. The visualized proteins are identified by mass spectroscopy and proteins whose activity lie downstream and are substrates for the p38 kinase are identified by the absence of the signal on the gel which separated lysates that were pre-treated with the specific kinase inhibitor.

11. For flow-through applications see the previous example for IFNA and ERB2.

12. For mass-spectrophotometric applications a Ciphergen Inc. mass spec. instrument was employed.

20 The resultant phosphoprotein-enriched eluates or whole cell extracts are incubated on bait traps that specifically bind phosphorylated proteins. Such baits may be protein-based (e.g., anti-phosphotyrosine antibody) or chemical (e.g., iron, gallium ion, etc.). The surfaces are 25 then washed and phosphorylated proteins detected and identified by time-of-flight or collision-induced daughter ion spectral analysis.

30 Novel circuit profiles and maps were successfully obtained using ligands that recognized the phosphorylated versions of the 20 proteins listed in above.

1. The following proteins were identified to lie downstream of p38 kinase: CREB, CD25, cJUN, Cdk1, and e1f2A.

35 2. The following proteins can be activated in primary human monocytes: EGFR, Tyk2, E1F2a, E1F4E, p38, CDK1, CREB, c-Jun, Elk-1, BAD, Jak1, Erk 1/2, CD25. The following were specific to monocytes and not found in lymphocytes from the same patient: EGFR, Tyk2, E1F2a.

- 23 -

3. The following proteins are specifically activated in primary human lymphocytes but not monocytes from the same patient: ERB2.

4. The following new unknown proteins changed  
5 their phosphorylation state in response to treatment with SB203589: mw. 100 kDa; mw. 85 kDa; mw. 35 kDa; mw. 30 kDa;  
mw. 25 kDa; mw. 20 kDa.

5. ERB2 positive primary human breast cancer cells show that ERK is interacting and binding with the  
10 following proteins to a greater level than ERB2 negative human breast cancer cells: pERB2, pERB1, pELK, p70S6K.

6. Fresh human brain and prostate tissue cells can be treated *ex vivo* with phosphatase inhibitors under the subject invention resulting in the identification of proteins  
15 that are phosphorylated in a tissue and disease specific manner.

FIG. 19 is a flow chart of a molecular detection method in accordance with the present invention. As indicated in step 402, the method includes arranging a series  
20 of recognition molecules or binding reagents which selectively bind to the various proteins, their phosphorylated or activated state, and their binding partners, of a signal transduction pathway to form a signal transduction pathway profiling chip 100 as described above by  
25 way of reference to FIG. 1. The method further includes applying (step 404) a cell lysate from a tissue of interest to the profiling chip. Thereafter, the sample is allowed to bind or hybridize with the recognition molecules at the binding sites 116 (FIG. 1) as indicated by step 406.

30 At step 408, binding events at the plurality of binding sites 116 are detected. The detection can be performed by a number of labeling or other techniques to produce a detectable signal, such as spectrophotometric, fluorometric, colorimetric, chemiluminescent, radiometric,  
35 electrochemical, photochemical, enzymatic, or optical readout techniques, and the like. In one embodiment, the step of detecting the binding events provides a qualitative indication of binding at each binding site. More preferably,

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however, a quantitative determination of binding at each binding site is made at step 408, for example, by determining an intensity or magnitude of the detectable signal at each binding site.

5 At step 410, the detected binding events are used to determine a characteristic of the analyzed sample. By identifying at which sites binding events occurred, the status of the selected pathway can be determined. The determination can be made by visual detection of the binding  
10 sites. Alternately, the characteristics can be determined automatically, for example, using a binding event sensor and pattern recognition software so that the pathway status can be determined under preprogrammed control. Furthermore, heuristic algorithms are used to correlate binding patterns  
15 and/or pathway status with cellular conditions, such as normal, pre-disease, or disease states. The use of patterns for the identification of a pathway and/or disease state of a cell sample is illustrated in FIGS. 20 and 21.

Appendix A of 17 pages is incorporated herein by  
20 reference. Appendix A presents further exemplary embodiments related to the protein interaction profiling system and method of the present invention.

The description above should not be construed as limiting the scope of the invention, but as merely providing  
25 illustrations to some of the presently preferred embodiments of this invention. In light of the above description and examples, various other modifications and variations will now become apparent to those skilled in the art without departing from the spirit and scope of the present invention as defined  
30 by the appended claims. Accordingly, the scope of the invention should be determined solely by the appended claims and their legal equivalents.

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CLAIMS

What is claimed is:

1. An assay device for use in determining the presence of a plurality of analytes in a lysate of a sample 5 of cells, the device comprising:  
(a) a porous support member; and  
(b) a plurality of binding reagents arranged and immobilized at a plurality of reaction sites on the support member, the binding reagents selected and arranged to assess the 10 status of a selected protein-protein interaction network when the lysate is applied thereto.

2. The device of claim 1, wherein the protein-protein interaction network is a signal transduction pathway.

3. The device of claim 2, wherein the binding 15 reagents are arranged to quantitatively assess the status of the selected signal transduction pathway.

4. The device of claim 2, wherein each binding reagent selectively binds with one of a series of biological molecules, the signal transduction pathway comprising said 20 series of biological molecules, the biological molecules selected from one or more proteins, a phosphorylated state of said one or more proteins, an activated state of said one or more proteins, and a binding partner of said one or more proteins.

25 5. The device of claim 1, wherein the protein-protein interaction network is relevant to a disease state.

6. The device of claim 5, wherein the disease state is selected from cancer, brain disease, cardiac disease, and an allergy.

30 7. The device of claim 2, wherein the binding reagents are ordered to at least partially recapitulate a

- 26 -

temporal or spatial status of molecular complex formations or networks of protein interaction which characterize the selected protein-protein interactive network.

8. The device of claim 7, wherein the binding  
5 reagents are bound in an immobilized flow-through matrix such that a plurality of protein complexes comprising different combinations of the same molecules can be sorted to determine the relative proportion of at least one selected molecule within the protein complexes.

10 9. The device of claim 7, wherein the protein-  
protein interactive network is branched.

10. The device of claim 7, wherein the protein-  
protein interactive network is a signal transduction pathway.

11. The device of claim 10, wherein the binding  
15 reagents are ordered to at least partially recapitulate the selected cellular signal transduction pathway in reverse temporal order.

12. The device of claim 10, wherein the binding  
reagents are ordered to at least partially recapitulate the  
20 selected cellular signal transduction pathway in forward temporal order.

13. The device of claim 10, wherein the binding  
reagents are ordered to at least partially recapitulate one  
25 of a signal pathway branch point and a connection to another pathway.

14. The device of claim 1, which comprises a kit for providing information about the therapeutic, toxic, or diagnostic state of a cellular sample.

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15. A method of elucidating complex protein-protein interactions within defined protein pathways and protein networks in cells, comprising:

- a) generating a lysate of cells containing one or more protein components of the protein-protein interactions;
- 5 b) applying the lysate to an immobilized series of binding reagents which discriminate the one or more protein components; and
- c) identifying binding events between the protein
- 10 components and the binding reagents.

16. A method for assessing the status of a selected signal transduction pathway in cells comprising:

- a) generating a lysate of cells containing components of signal pathway proteins, the lysate retaining, at least in part, one or more pathway molecules, the pathway molecules present in one or more states selected from inactive, activated, activity altered, phosphorylated, cleaved, modified, and bound, the one or more pathway molecules reflecting signal transduction events taking place in the cells;
- 15 b) applying the lysate to an immobilized series of binding reagents which discriminate the one or more pathway molecules and the one or more states thereof;
- c) identifying binding events between the pathway molecules
- 20 and the binding reagents; and
- d) determining the state of the selected signal pathway.

17. The method of claim 16, wherein the immobilized binding reagents are ordered to at least partially recapitulate a temporal and or spatial status of molecular complex formations or networks of protein interaction which characterize the selected cellular signal pathway.

18. The method of claim 16, wherein the immobilized binding reagents are arranged in a preselected arrangement and wherein the determining step comprises

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identifying a pattern of binding events based on said arrangement to form a pattern of binding events.

19. The method of claim 18, wherein the determining step further comprises using a heuristic to  
5 provide information about the temporal or spatial status of cellular pathways and networks.

20. The method of claim 18, wherein the heuristic is selected from one or both of a heuristic-based bioinformatic and a genetic algorithm.

10 21. The method of claim 18, wherein the determining step further comprises comparing the identified patterns of binding events with one or more previously identified patterns for cells of a known condition.

22. The method of claim 21, wherein the binding  
15 reagents are bound in an immobilized flow-through matrix such that protein complexes comprising different combinations of the same molecules can be sorted to determine the relative proportion of at least one chosen molecule within different complexes.

20 23. The method of claim 22, wherein a signal pathway is at least partially recapitulated in reverse temporal order.

24. The method of claim 22, wherein a signal pathway is at least partially recapitulated in forward  
25 temporal order.

25. A method of claim 22, wherein a signal pathway branch point or connection to another pathway is at least partially recapitulated.

26. The method of claim 16, wherein the signal  
30 pathway is a pathway selected from one of the following

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classes: cell growth, cell differentiation, cell death, cell movement, gene transcription regulation, hormonal autocrine or paracrine stimulation, and cell adhesion pathways.

27. A method for determining the state of at least  
5 a portion of a signal pathway or network in a cell,  
comprising:

solubilizing the cellular contents;  
exposing the contents to a series of labeled  
binding reagents to form a pattern of binding events; and  
10 analyzing the pattern of binding events.

28. The method of claim 27, wherein the binding  
reagents comprise ligands selected from one or more of  
antibodies, nucleic acids, synthetic molecules, drugs,  
bacteriophage, engineered prokaryotic cells, and engineered  
15 eukaryotic cells.

29. The method of claim 27, in which the labeled  
binding reagents generate a signal via chemiluminescence,  
fluorescence, radiometric, electrochemical, photochemical,  
enzymatic, colorimetric, or optical readout.

20 30. The method of claim 28, wherein the binding  
reagents are immobilized on a planar surface.

31. The method of claim 30, wherein the binding  
reagents are arranged in one of a linear array and a two-  
dimensional grid.

25 32. The method of claim 28, wherein binding  
reagents are generally linearly arranged and the cell  
contents are passively or actively made to flow through  
multiple binding zones.

33. The method of claim 28, wherein the binding  
30 reagents are arranged in a three-dimensional matrix.

- 30 -

34. The method of claim 33, wherein the matrix comprises a circuit containing branch-points in a flow of molecular complexes within the said matrix.

35. The method of claim 33, wherein molecules bound in one zone are released during a flow state and the released molecules can bind to a separate zone downstream in the flow.

36. A method for identifying proteins involved in cellular signaling or networks comprising:

- 10 a) exposing cells to one of a phosphatase inhibitor followed by a drug such that the phosphorylation state of one or more molecules is changed;
- b) analyzing patterns of groups of at least 2 phosphorylated molecules before and after exposure to the drug;
- c) exposing cells to a molecule which perturbs one or more pathways; and
- d) comparing the patterns before and after exposure of the cells to said molecule.

20 37. The method of claim 36, further including the step of:

- (e) identifying therapeutic targets by evaluating whether a candidate compound either binds to a phosphorylated protein or perturbs the pattern of groups of at least 2 phosphorylated proteins.

38. A method of claim 36, which is used to determine at least one of therapeutic efficacy and toxicity of a preselected molecule being evaluated for therapeutic potential.

30 39. The method of claim 15, further comprising identifying one or both of drug-induced toxicity and therapeutic efficacy by evaluating one or more patterns of protein-protein interactions.

- 31 -

40. The method of claim 39, wherein the patterns are evaluated through heuristically-based and/or genetic algorithm-based data mining.

41. The method of claim 16, further comprising  
5 identifying one or both of drug-induced toxicity and therapeutic efficacy by evaluating one or more patterns of protein-protein interactions.

42. The method of claim 41, wherein the patterns are evaluated through heuristically-based and/or genetic  
10 algorithm-based data mining.

43. A method of identifying a repertoire of proteins that serve as acceptors for phosphorylation, comprising:

- (a) treating a first biological sample with one or  
15 more compounds that inhibit one or both of protein tyrosine and serine/threonine phosphatase activity;
- (b) isolating and lysing cells of interest;
- (c) selecting and enriching for phosphorylated proteins using antibodies on an immobilized bait to produce  
20 an enriched fraction;
- (d) separating the phosphoproteins in the enriched fraction;
- (e) identifying a primary amino acid sequence of the separated proteins; and
- 25 (f) identifying binding molecules that specifically bind to the separated proteins.

44. The method of claim 43, wherein the biological sample is selected from cells, whole tissue specimens grown ex-vivo, and animal tissue treated in vivo.

30 45. The method of claim 43, wherein the compound that inhibits one or both of protein tyrosine and serine/threonine phosphatase activity is selected from one or more of sodium pervanadate, okadaic acid, calyculin A.

- 32 -

46. The method of claim 45, wherein the biological sample is treated for a period of time that is less than about 3 hours.

47. The method of claim 43, wherein the antibodies 5 are selected from one or more of anti-phosphotyrosine, anti-phosphoserine, and anti-phosphothreonine.

48. The method of claim 43, wherein the phosphoproteins are separated by one or both of a chromatographic techniques and an electrophoretic technique.

10 49. The method of claim 43, wherein the sequence identifying step comprises:

degrading the protein by one of enzymatic digestion and chemically-induced protein fragmentation;

identifying the sequence using mass spectrometry.

15 50. The method of claim 43, wherein the binding molecules are antibodies.

51. The method of claim 43, wherein the step of separating the phosphoproteins in the enriched fraction is performed by applying the lysate to an assay device 20 comprising a porous support member and a plurality of binding reagents arranged and immobilized at a plurality of reaction sites on the support member.

52. The method of claim 43, wherein the step of separating the phosphoproteins comprises:

25 applying the lysate to an immobilized series of binding reagents which discriminate the one or more protein components; and

identifying binding events between the protein components and the binding reagents.

30 53. The method of claim 43, further comprising:

- 33 -

- (g) forming an immobilized arraying said binding molecules;
- (h) treating a second biological sample with a drug; and
- 5 (i) evaluating one or both of drug-induced toxicity and drug efficacy by identifying binding events between the immobilized array and proteins in the biological sample.

54. The method of claim 53, wherein the step of  
10 evaluating further comprises identifying a pattern of the binding events.

55. The method of claim 43, wherein the biological sample comprises a whole tissue sample, further comprising:  
fixing the tissue sample;  
15 selecting a portion of the tissue sample; and  
identifying a cellular localization of an identified phosphorylated protein.

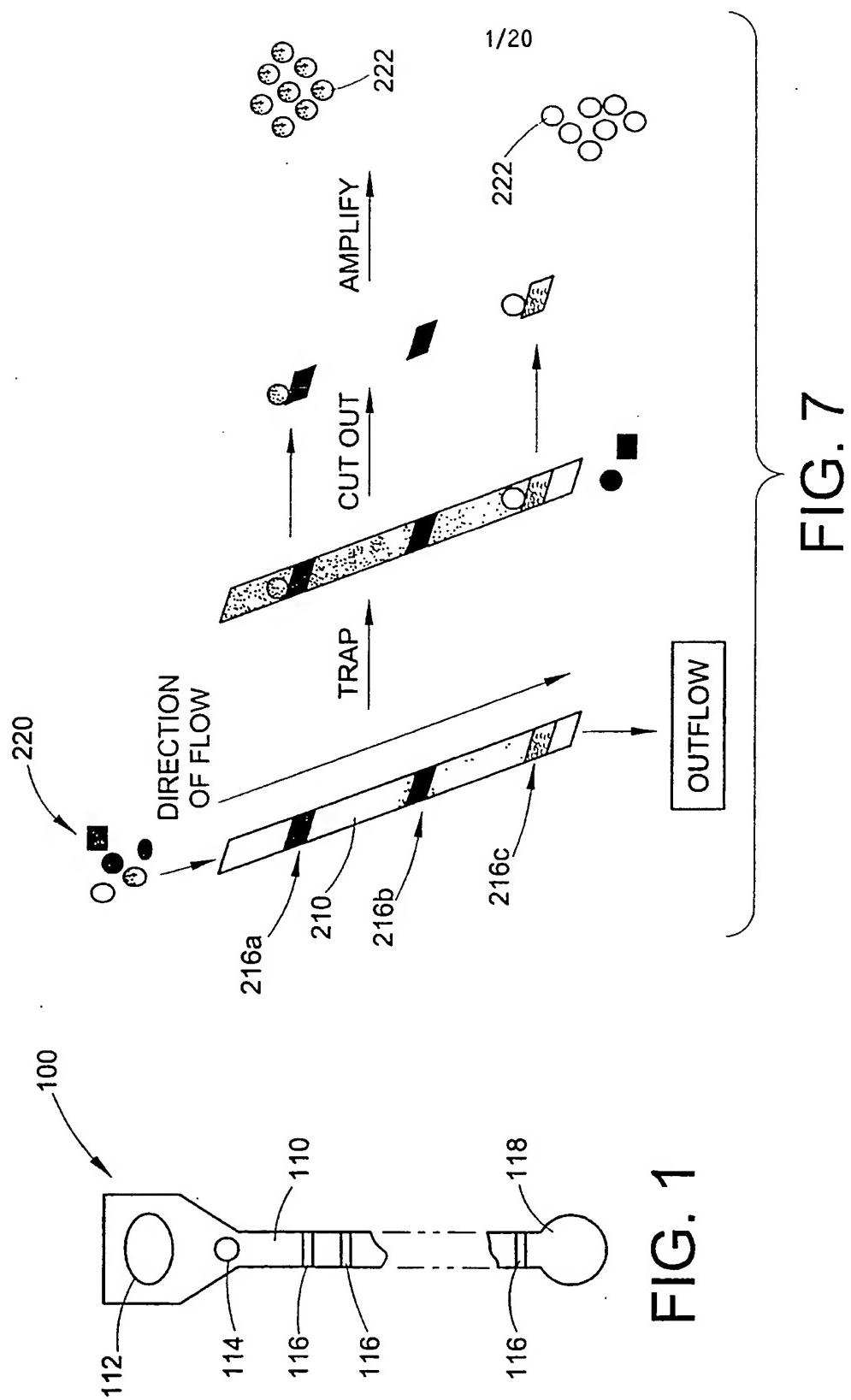


FIG. 1

FIG. 7

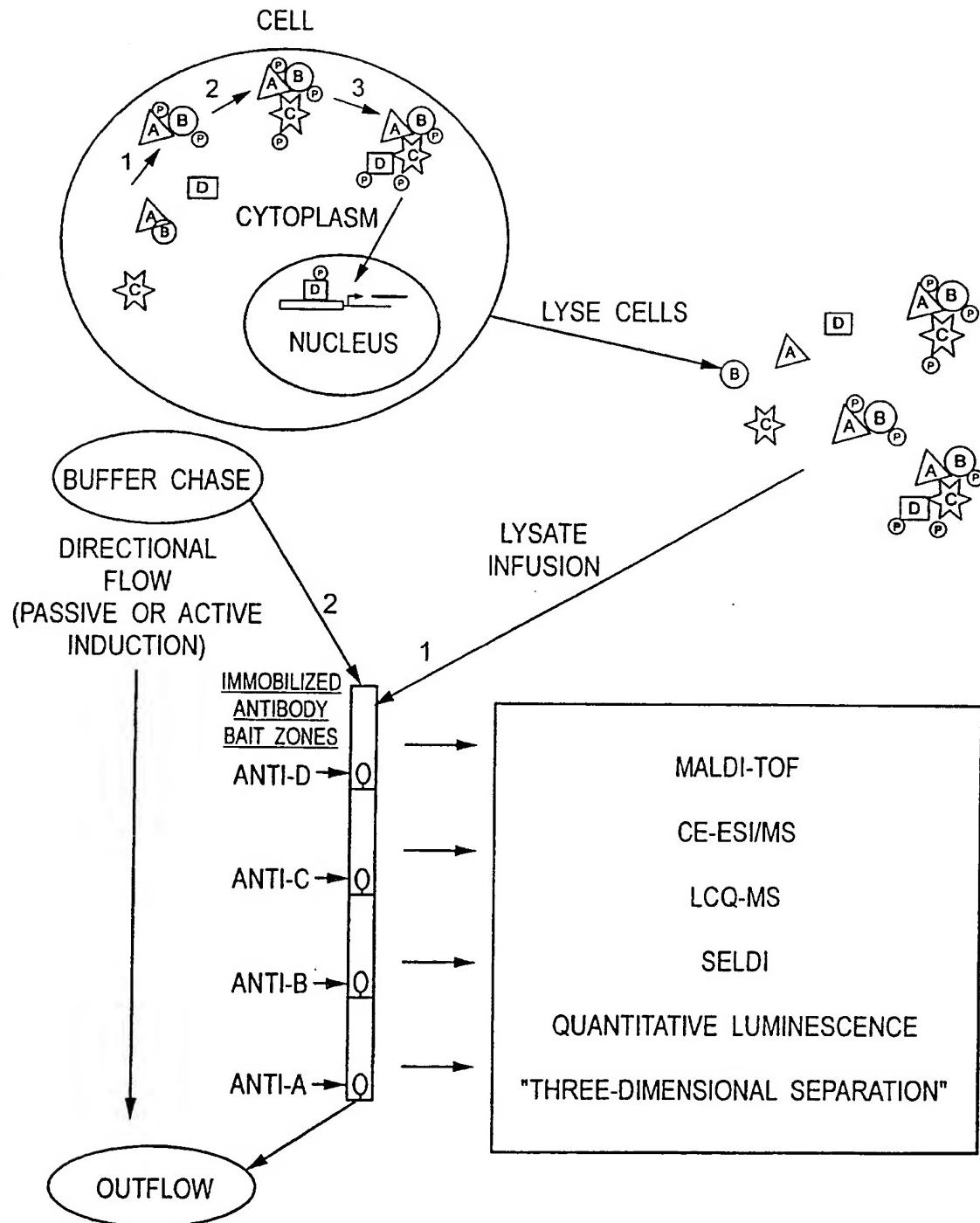
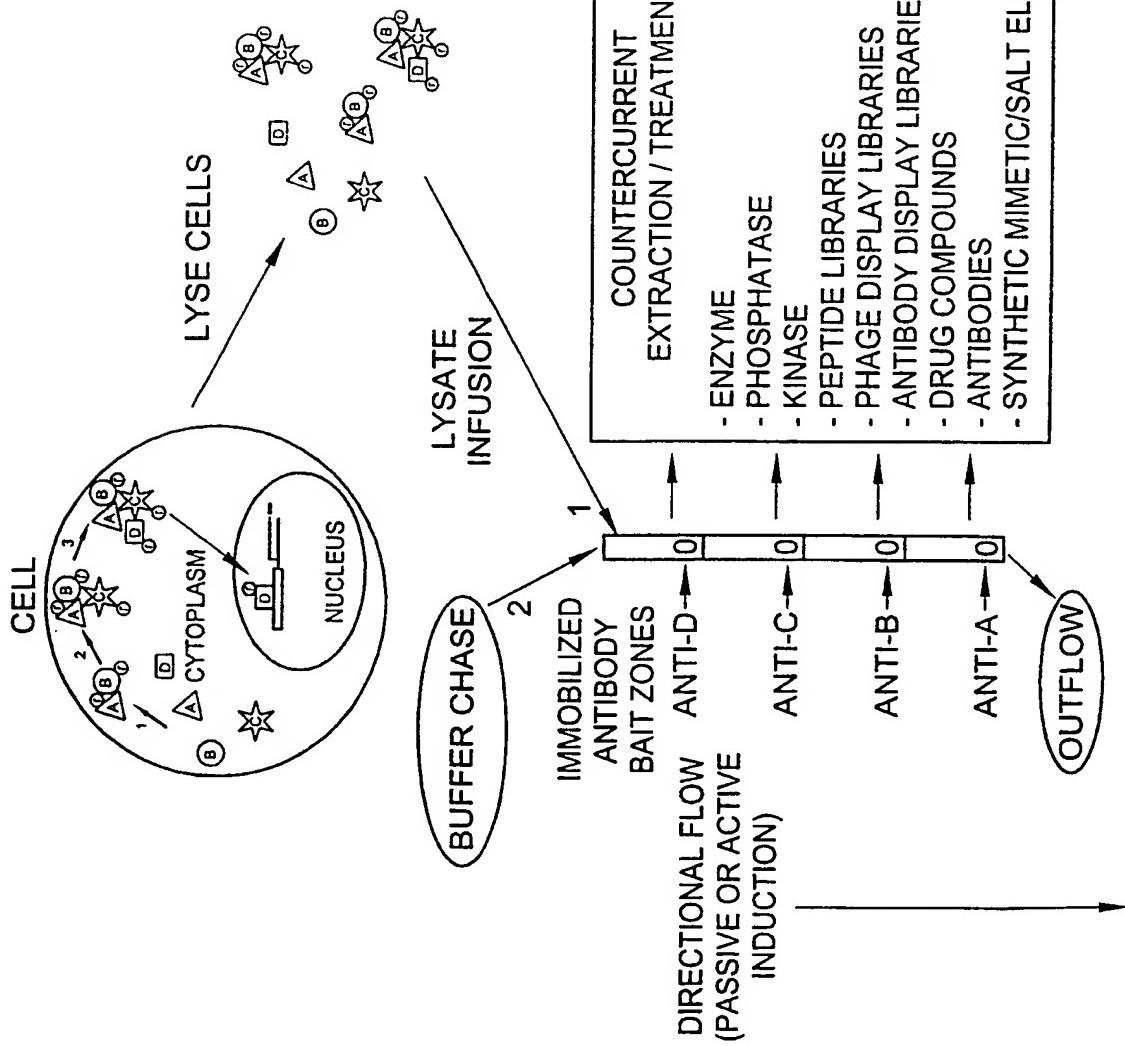


FIG. 2

FIG. 3



4/20

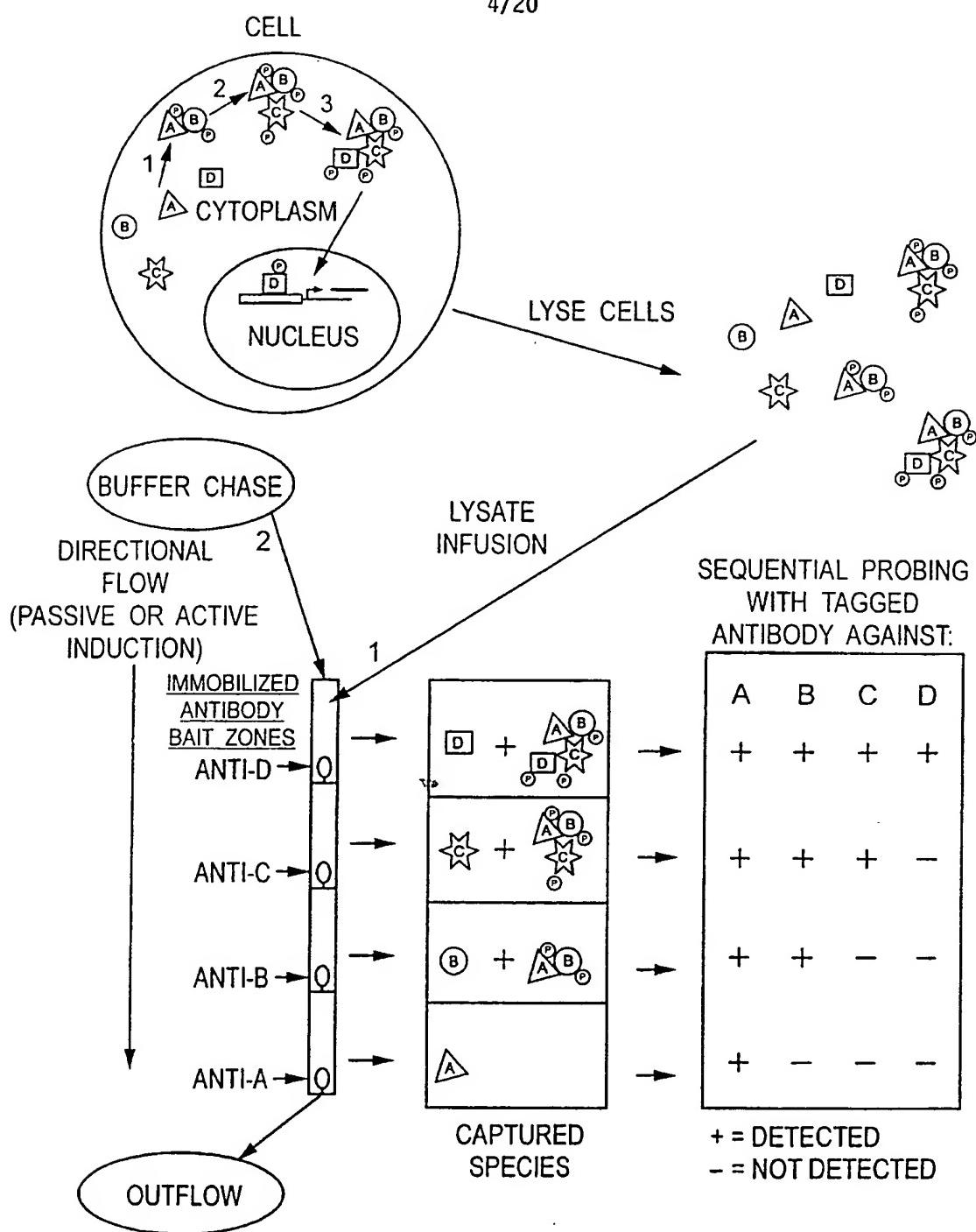
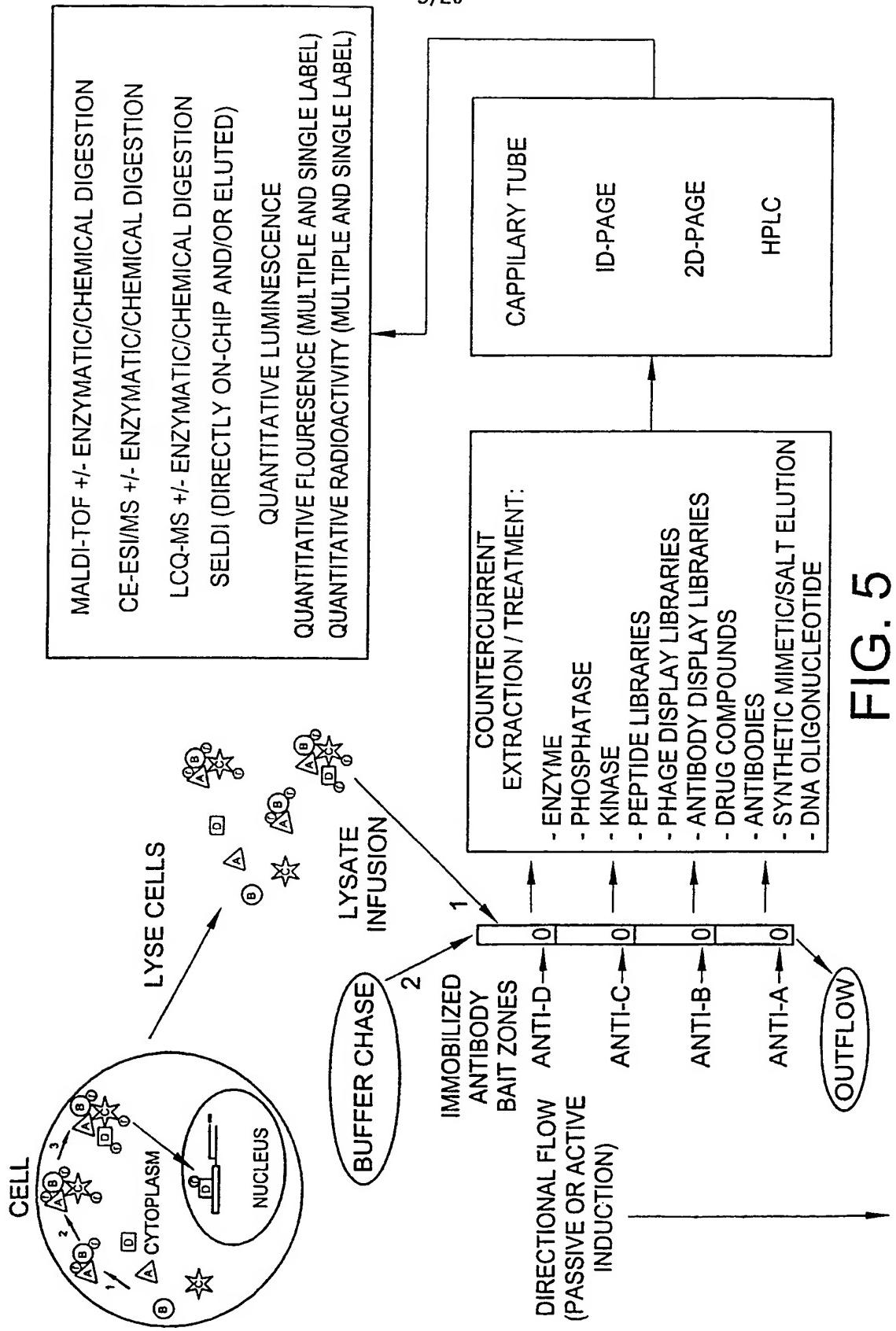


FIG. 4

**FIG. 5**

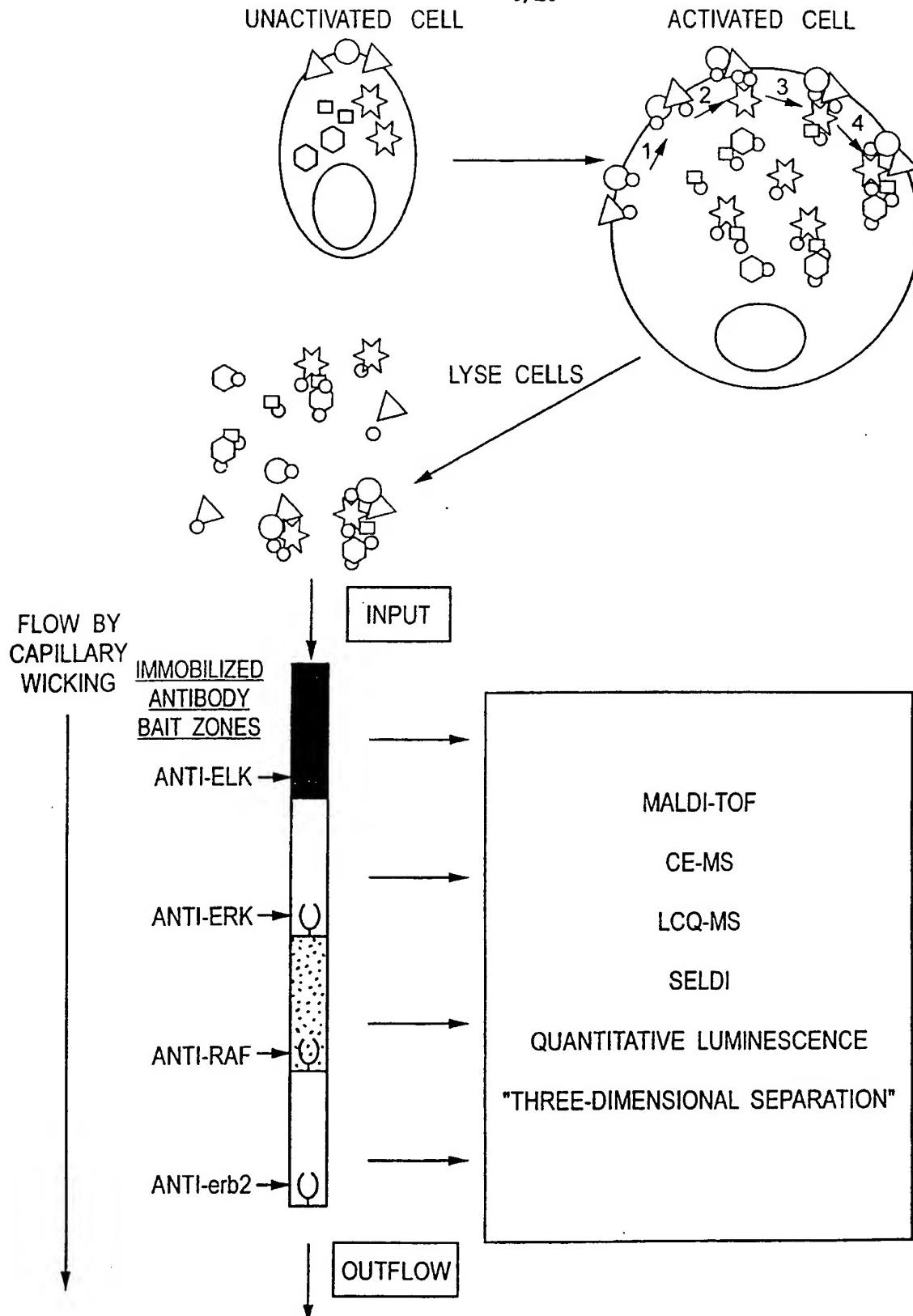


FIG. 6

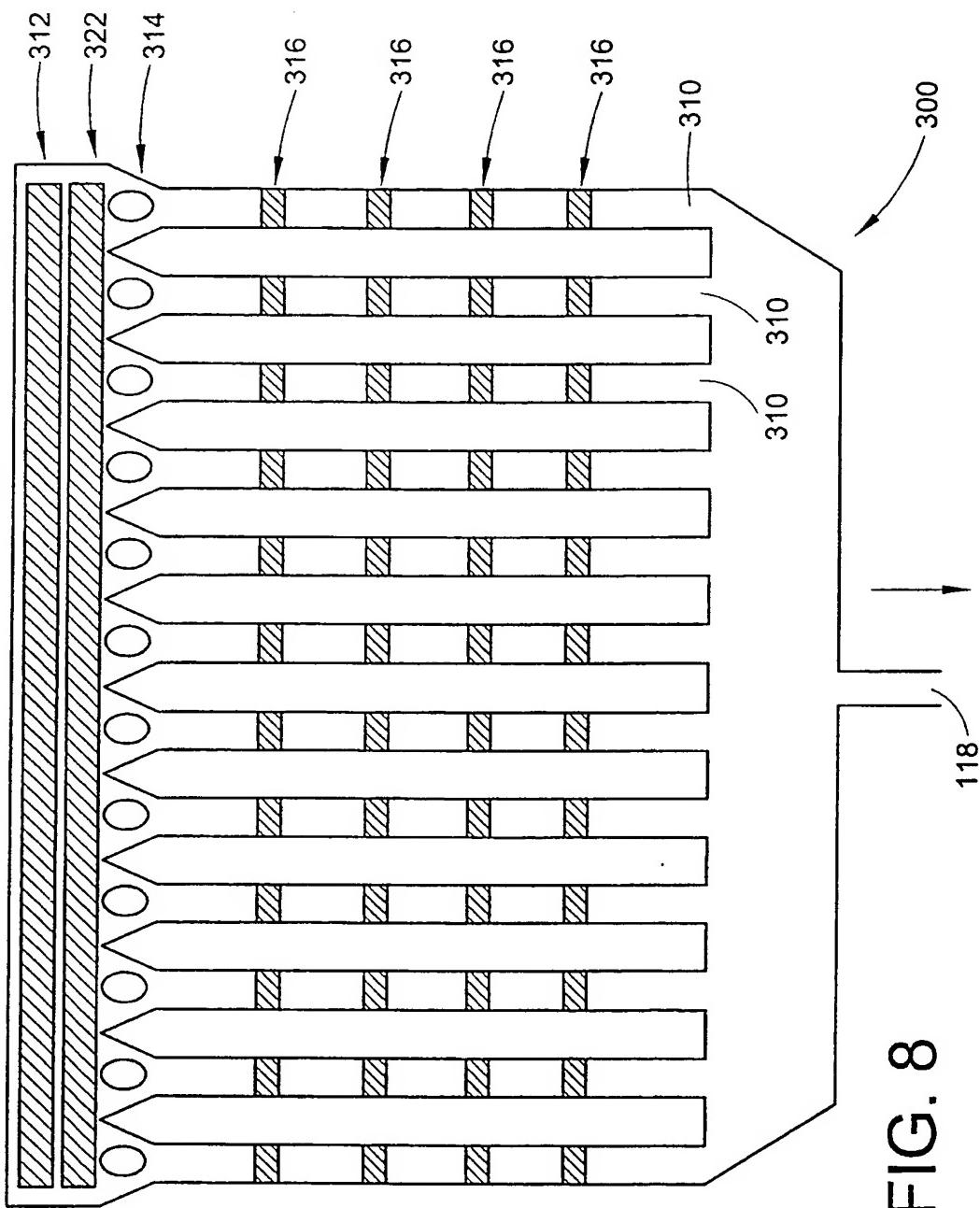


FIG. 8

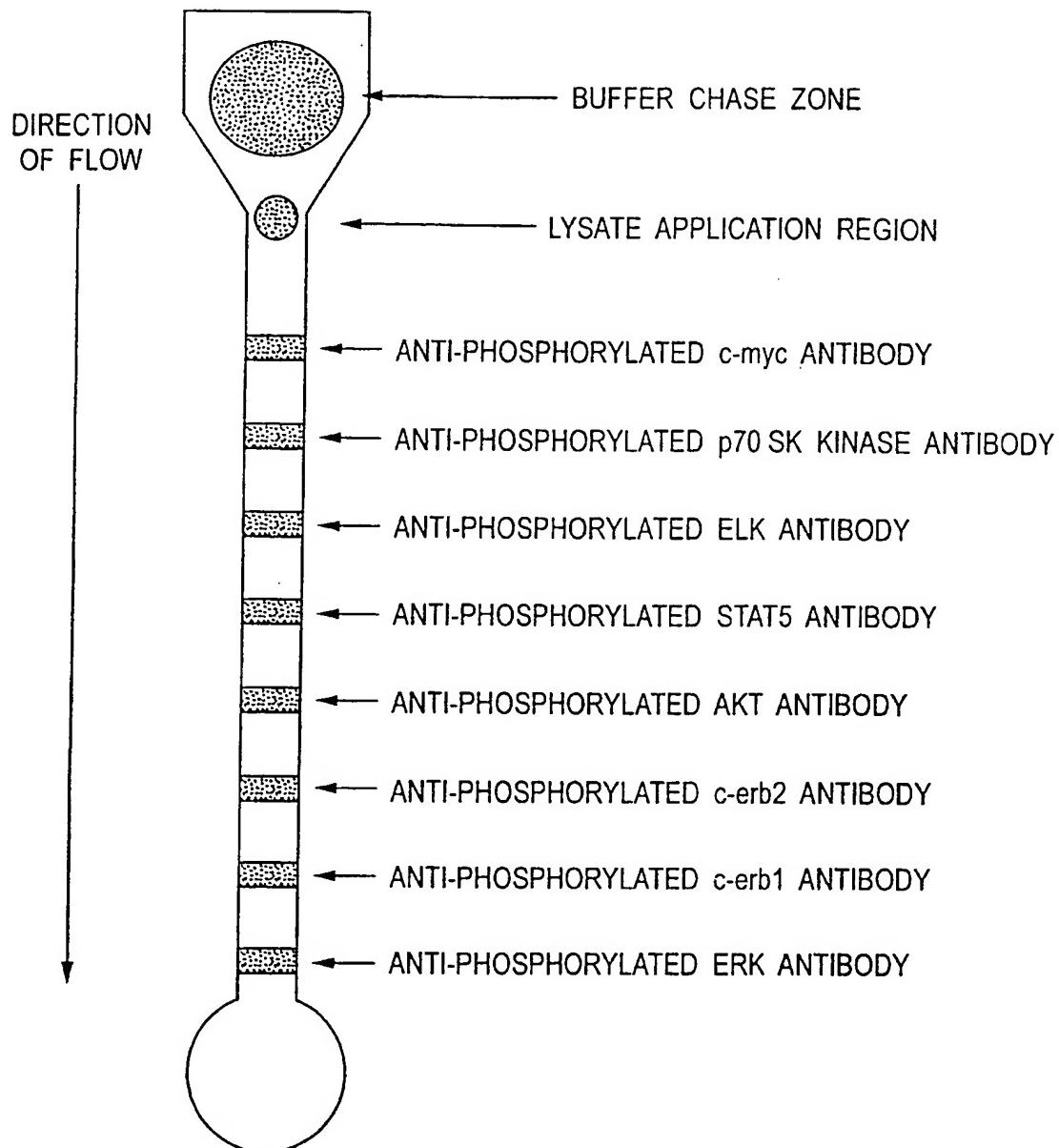


FIG. 9

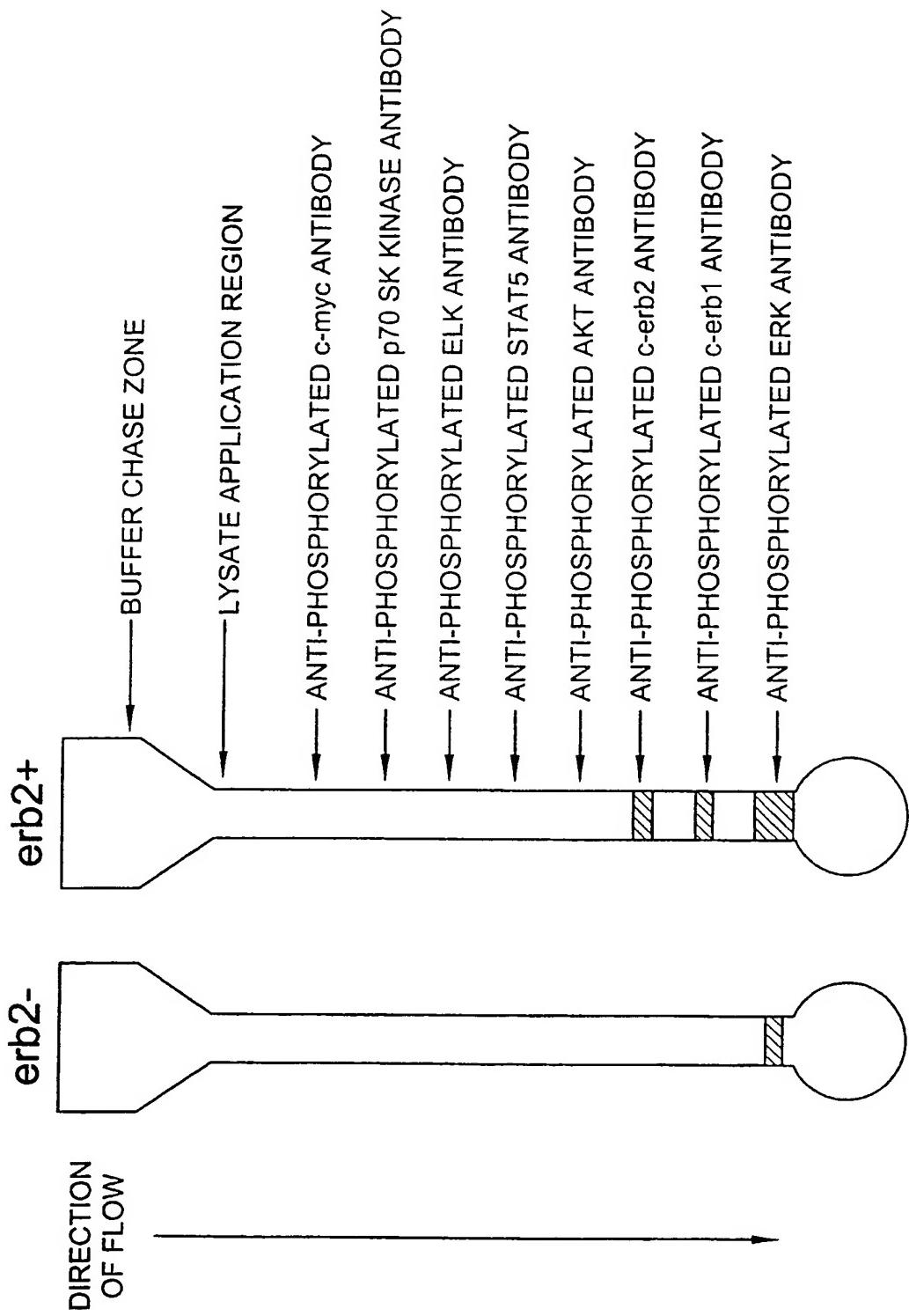


FIG. 10

10/20

$\alpha$  - INTERFERON TREATMENT  
SAMPLE: PERIPHERAL BLOOD LEUKOCYTES  
PRIMARY ANTIBODY / PROBE: p-STAT1

0 MINUTE    3 MINUTES    10 MINUTES    30 MINUTES

Stat2    [blot]    [blot]    [blot]    [blot]

Stat1    [blot]    [blot]    [blot]    [blot]

cts/s

Stat2    0    169.4    141.7    49.4

Stat1    0    83.9    118.2    61.7

FIG. 11

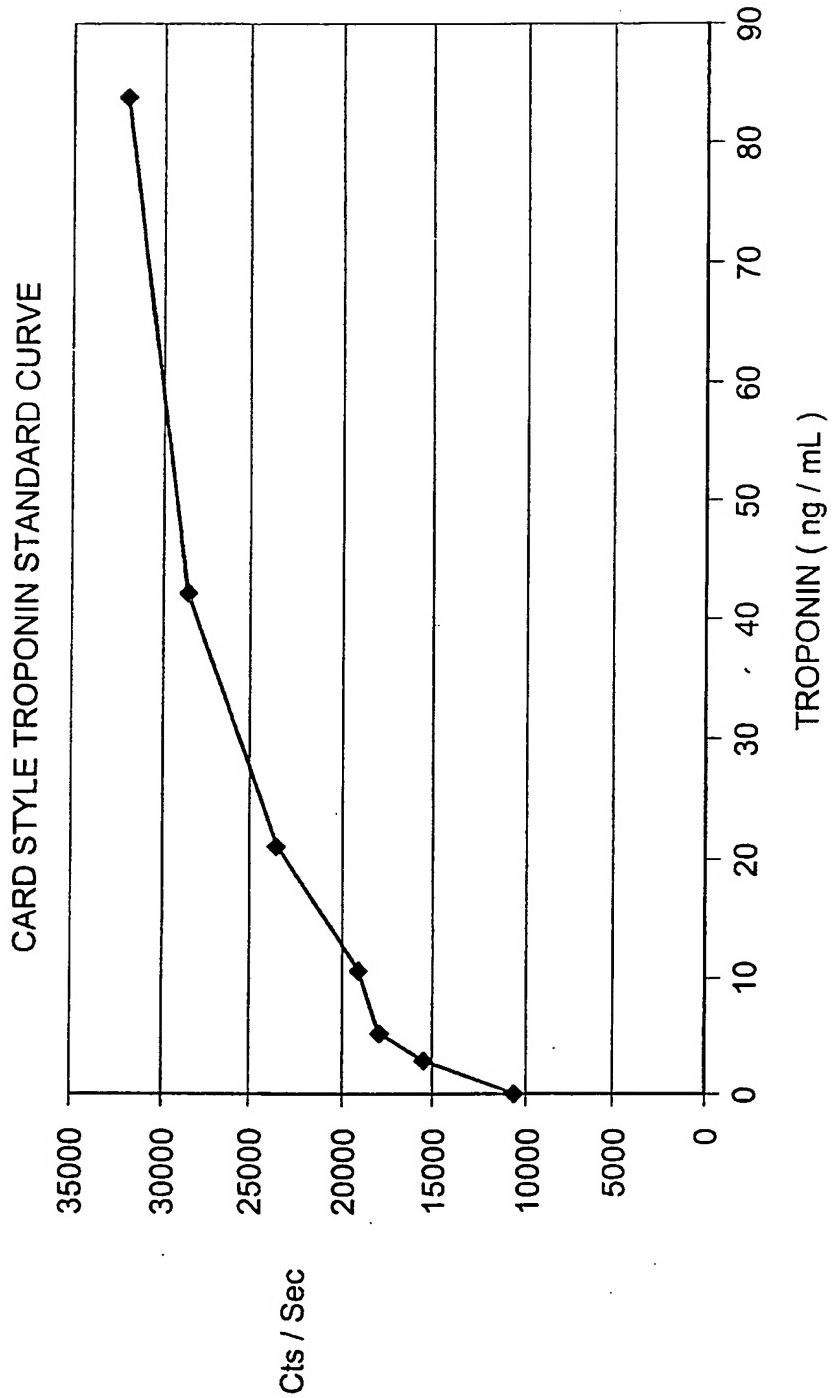


FIG. 12

12/20

CPG BEADS CONJUGATED TO HRP  
SUBSTRATE: SUPERSIGNAL

LIGHT OUTPUT ZONES SEPARATED  
BY UNMODIFIED CPG BEADS  
1 SECOND / HIGH RESOLUTION PACKED COLUMN

LIGHT OUTPUT OF "PLUG FORMAT"  
1 SECOND / HIGH RESOLUTION

PHOTO OF ZONES SEPARATED BY PLUGS

OVERLAY OF "PLUG FORMAT"

## FIG. 13A

CPG BEADS CONJUGATED TO P14-Thy  
CONJUGATE: P3-AP (1:500)  
SUBSTRATE: DuoLux-AP

LIGHT OUTPUT 1 SECOND / HIGH RESOLUTION

PHOTO OF DRUMMOND CAPILLARY

OVERLAY

## FIG. 13B

CPG BEADS CONJUGATED TO P14-Thy  
CONJUGATE: P3-HRP (1:1000)  
SUBSTRATE: DuoLux-AP

LIGHT OUTPUT 1 SECOND / HIGH RESOLUTION

PHOTO OF DRUMMOND CAPILLARY

OVERLAY

## FIG. 13C

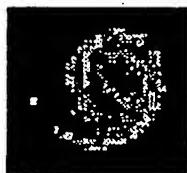
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PCT/US01/03535

13/20

SENSITIVITY OF CPG BEADS



BACKGROUND  
CTS/S 423



1 HRP CPG BEAD  
1424

FIG. 14A

SPECIFICITY OF BINDING TO CPG BEADS  
SAMPLE: 10ug/mL p-STAT1 PEPTIDE: BSA  
PROBE: p-Tyr (RC20): AP



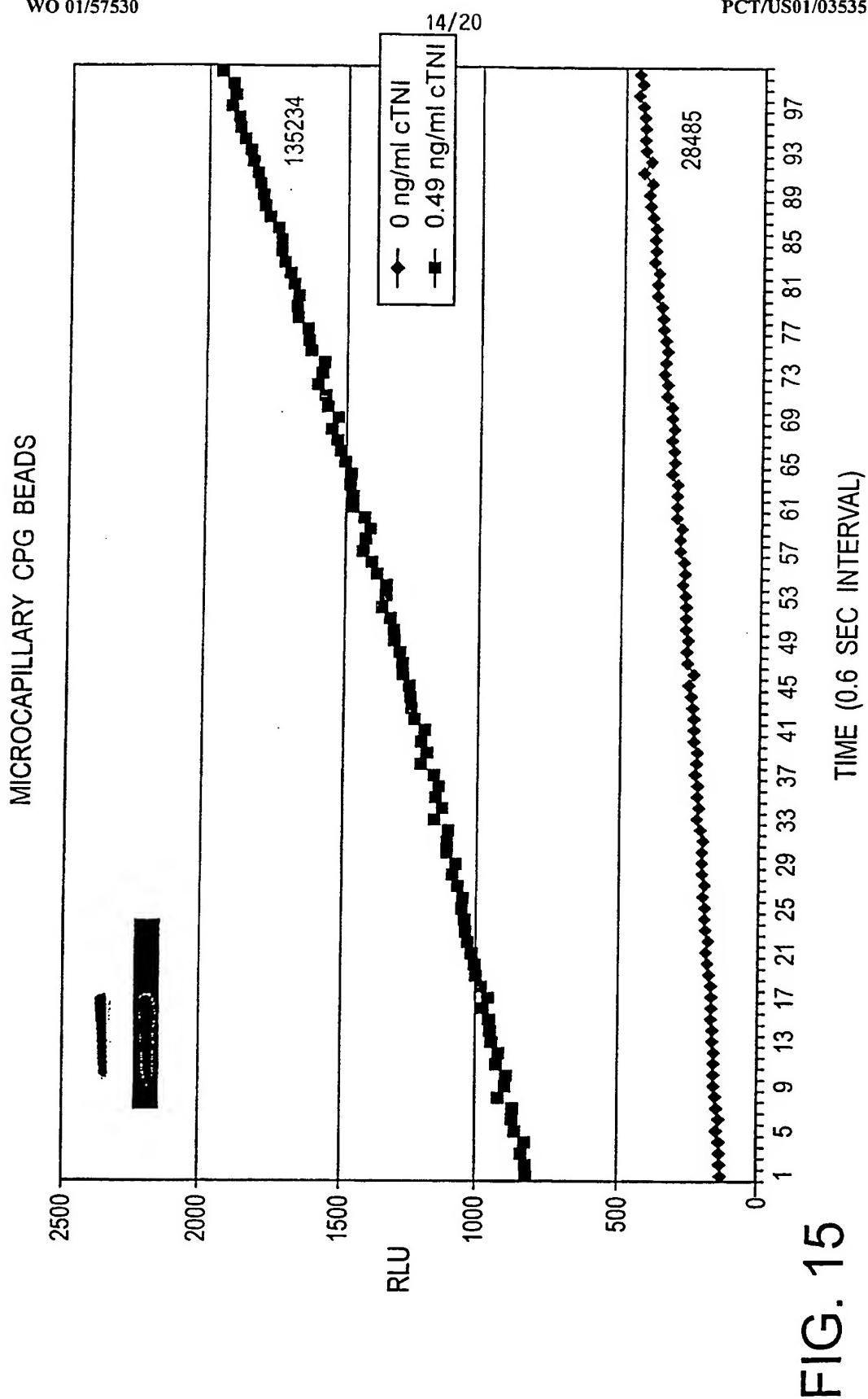
STAT 1            p-STAT 1  
CTS/S            15905            18430

PEPTIDE DISPLACEMENT STUDY  
p-STAT 1 CPG BEADS  
BLOCKING PEPTIDE: 10ug/mL p-STAT 1 PEPTIDE: BSA  
PROBE: p-STAT 1 PEPTIDE: HRP



-PEPTIDE            +PEPTIDE  
CTS/S            16200            10099            CORRECTED

FIG. 14B



15/20

STAT 1 CPG BEADS  
SAMPLE: INF-ALPHA TREATED PBL'S (50uL)  
PROBE: p-TYR(RC20): AP  
SUBSTRATE CDP-STAR

## JUNIOR DATA

NEGATIVE	36571
7 MIN RE-RUN	39712
BEAD WASH	4857
POSITIVE	34100
7 MIN RE-RUN	409185

## NIGHT OWL DATA

NEGATIVE [REDACTED] 135 CTS/S

POSITIVE [REDACTED] 313 CTS/S

FIG. 16

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*BEST AVAILABLE COPY*

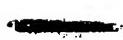
CPG STAT1 ANTIBODY BEADS  
PRIMARY ANTIBODY: p-Tyr  
SECONDARY ANTIBODY: GAM-AP  
SAMPLE: INTERFERON ALPHA TREATED PBL's

NEGATIVE



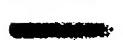
POSITIVE

NEGATIVE



572 cts/s

POSITIVE



1202 cts/s

FIG. 17

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**BEST AVAILABLE COPY**

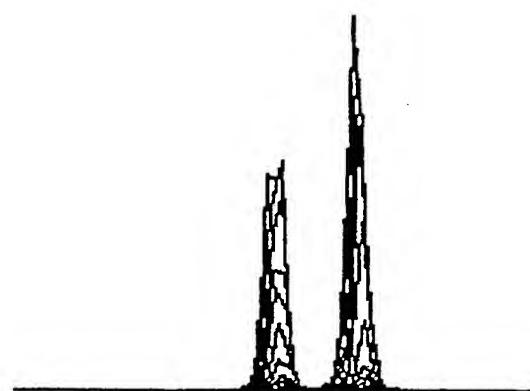
CPG STAT 1 ANTIBODY BEADS IN Z-SPIN COLUMNS

PRIMARY ANTIBODY: p-TYR

SECONDARY ANTIBODY: GAM-AP

SUBSTRATE: CDP-STAR

SAMPLE: PERVANADATE TREATED PBL'S

NEUTRAVIDIN  
BEADSSTAT 1 AB  
BEADS**FIG. 18**

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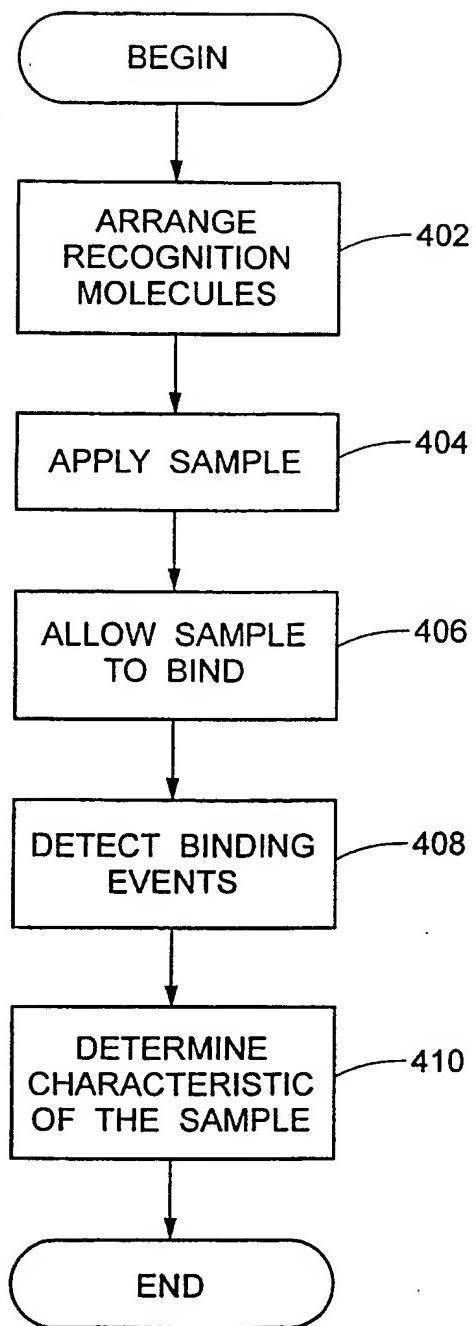


FIG. 19

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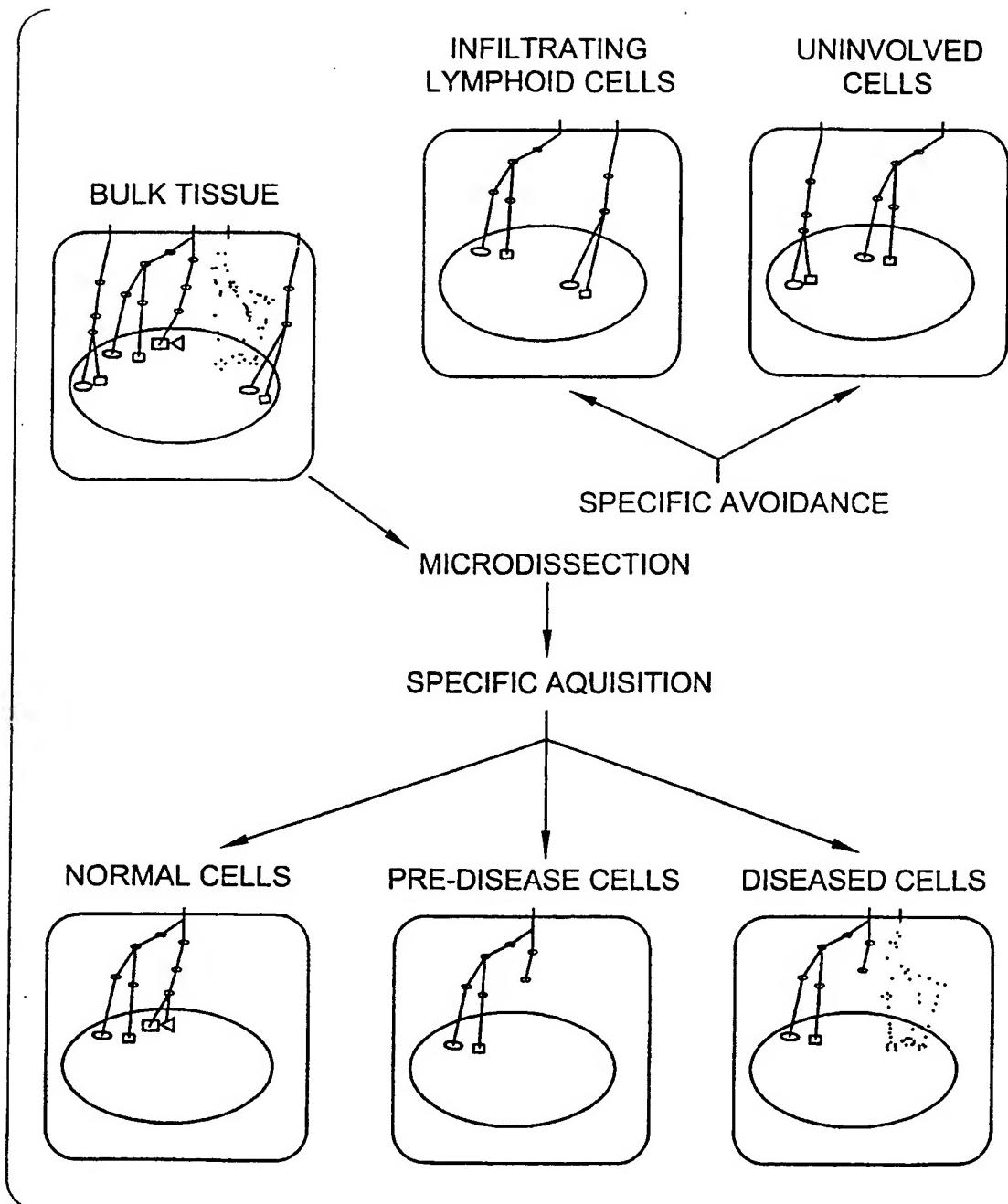


FIG. 20

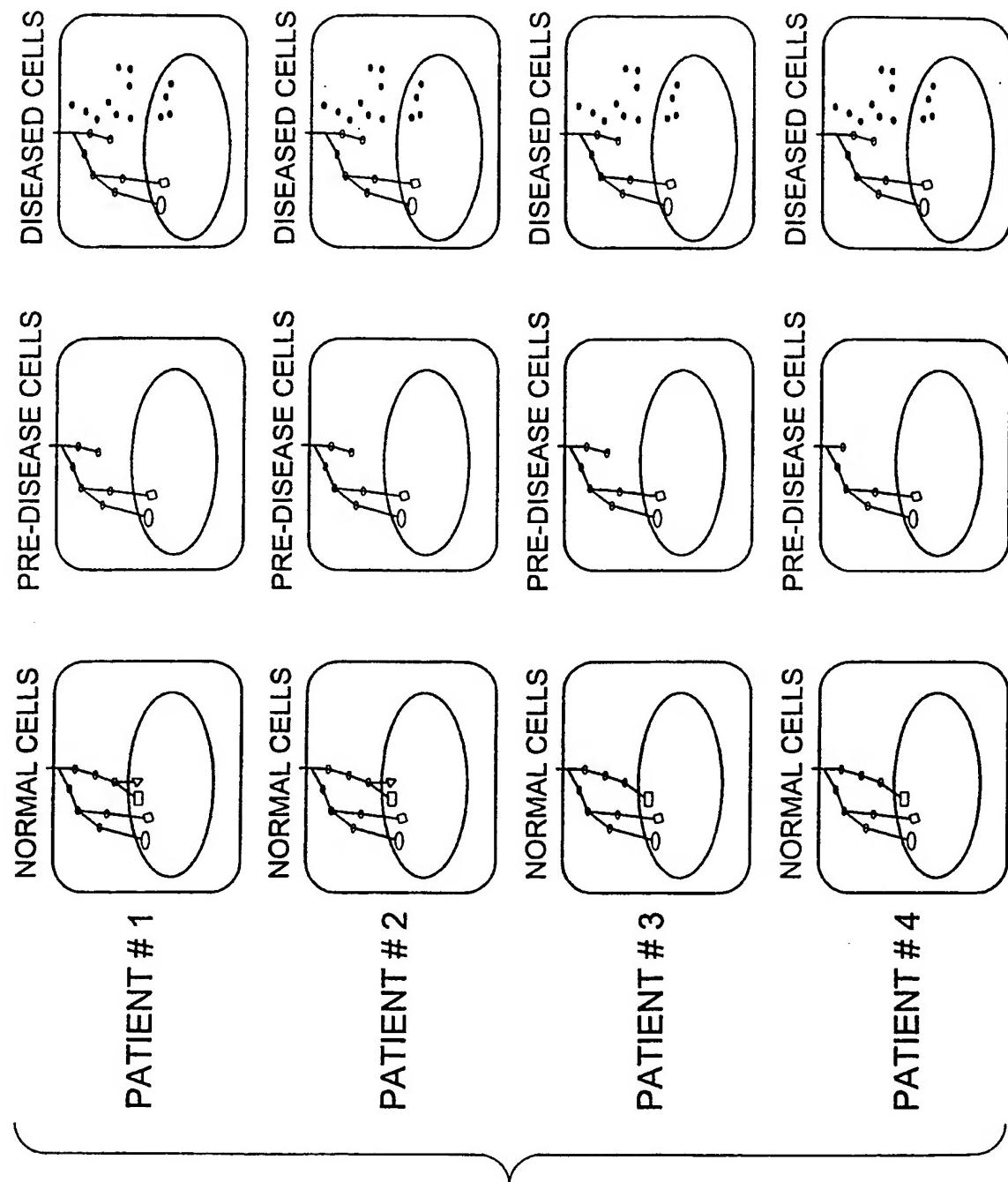
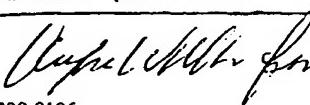


FIG. 21

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US01/03535

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC(7) : G01N 33/53, 33/543 US CL : 435/7.1; 436/518 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/7.1, 6, 287.1, 287.2; 436/518, 524, 528		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,789,154 A (DURST ET AL.) 04 August 1998 (04/08/1998), Figures 1-3; and column 10, line 21 to column 17, line 9.	1-55
Y	US 5,580,794 A (ALLEN) 03 December 1996 (03/12/1996), Figures 6-21; column 3, line 64 to column 4, line 57; and examples.	1-55
Y	US 5,958,791 A (ROBERTS ET AL.) 28 September 1999 (28/09/1999), Figures 1 and 3; and column 5, line 29 to column 8, line 38.	1-55
A	US 5,670,322 A (EGGERS ET AL.) 23 September 1997 (23/09/1997).	1-55
A	US 5,252,743 A (BARRETT ET AL.) 12 October 1993 (12/10/1993).	1-55
A	US 5,624,711 A (SUNDBERG ET AL.) 29 April 1997 (29/04/1997).	1-55
Y	WO 99/40434 A1 (INVITROGEN) 12 August 1999 (12/08/1999), page 14, line 22 to page 17, line 28.	1-55
Y	ROWE ET AL. Array biosensor for simultaneous identification of bacterial, viral, and protein analytes. Anal. Chem. 1 September 1999, Vol. 71, No. 17, pages 3846-3852, especially pages 3847-3849, experimental section.	1-55
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 03 April 2001 (03.04.2001)		Date of mailing of the international search report <b>30 April 2001 (30.04.01)</b>
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230		Authorized officer Minh-Quan K. Pham  Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/03535

## C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DZGOEV ET AL. Microformat imaging ELISA for pesticide determination. Anal. Chem. 1 October 1996, Vol. 68, No. 19, pages 3364-3369, especially pages 3365-3366, experimental section.	1-55
Y	EKINS ET AL. Multianalytic microspot immunoassay - Microanalytical "compact disk" of the future. Clin. Chem. 1991, Vol. 37, No. 11, pages 1955-1967, especially pages 1960-1966.	1-55
Y	EKINS ET AL. Multianalytic immunoassay: the immunological "compact disk" of the future. J. Clin. Immunoassay. 1990, Vol. 13, No. 4, pages 169-181, especially pages 173-179.	1-55
Y	EKINS. Ligand assays: from electrophoresis to miniaturized microarrays. Clin. Chem. 1998, Vol. 44, No. 9, pages 2015-1030, especially pages 2024-2029.	1-55